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From the Cytogenetics Laboratory, Department of Botany, Calcutta University.

ORCEIN STAINING AND THE STUDY OF THE EFFECT OF CHEMICALS ON CHROMOSOMES.

By

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With 12 figures in the text.

(Eingegangen am 23. April 1954/10. Februar 1955.)

Introduction.

The study of chemical mutagens has become a fascinating subject in recent years for cytochemists (Levan 1949, Levan and Tjio 1948, d'Amato 1950, Oehlkers 1953). Among the chemicals studied, several dyes have been found to possess chromosome-breaking properties (d'Amato 1950, 1951).

Different theories have been proposed as to the mode of action of mutagenic agents (Levan and Tjio 1948, Gray 1953, Koller 1948). For oxyquinoline (Tjio and Levan 1950, Sharma and Ghosh 1950, Sharma and Bhattacharjee 1952, Stålfelt 1950) and coumarin (Sharma and Bal 1953) which are used to obtain well defined chromosome plates, the effect is believed at least by the majority of the authors to be physical. It is, however, quite apparent that fragmentation can not be produced by entirely physical means. Some chemical changes affecting the skeleton of the chromosome are in all probability involved.

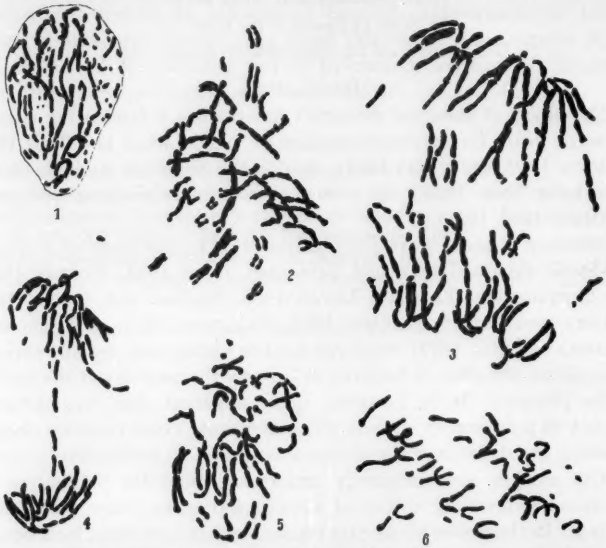
Our studies were originally undertaken in order to analyse the chromosome-breaking ability of alloxan and a few other purine derivatives. In the course of our experiments certain important facts became apparent which, we think, should be carefully considered before attacking any such problem.

Procedure for demonstrating fragmentation by orcein.

The schedule generally followed in the study of the effect of chemicals consists in treatment of root-tips by the chemical followed by fixation in acetic-alcohol and staining in aceto-orcein or by the Feulgen procedure. In some cases fixation in acetic-alcohol is omitted, especially when the chemical concerned acts itself as a fixative. The tissue then is softened by hydrolysis for a specified period in N/HCl in the Feulgen technique, and by heating over a flame for a few seconds in the acid-dye mixture in the aceto-orcein technique.

To investigate the effect of alloxan on chromosomes, root-tips of both monocotyledonous and dicotyledonous plants (*Allium, Hydrilla, Amaryllis, Lens, Lathyrus, Pisum* etc.) were brought into the solution for

1 to 3 hours. This treatment was followed by softening in the dye-acid mixture (2% aceto-orcein 9 parts + N/HCl 1 part) and staining in aceto-orcein. Considerable clumping was noted in all slides prepared by this method. Thus, application of coumarin (Sharma and Bal 1953) becomes necessary for a better definition of the chromosome structure. Improvements were obtained by this modification. Therefore all materials were subsequently treated in a saturated aqueous solution



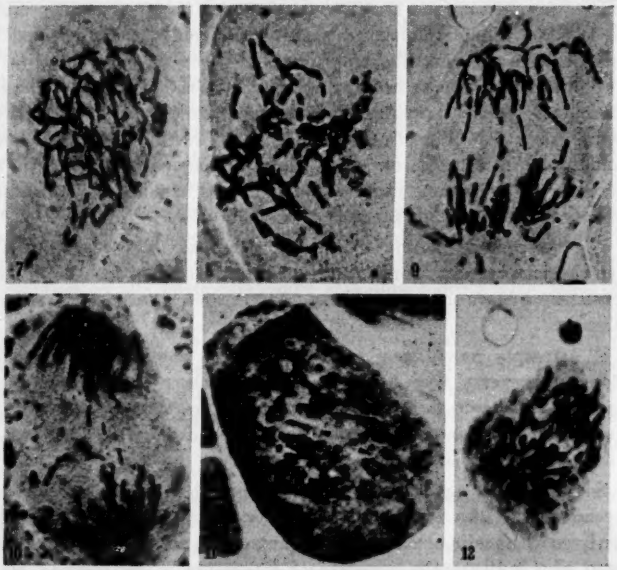
Figs. 1—6. Fragmentation of chromosomes in root-tip cells of Allium cepa after heat treatment in aceto-orcein solution. 1—4, prophase, metaphase, anaphase, and early telophase respectively showing the presence of numerous fragments after treatment in alloxan and coumarin. In 2 chromosomes longitudinally split, in 3 erosion noticeable. 5, 6, early metaphase and anaphase, fragments present after pretreatment in alloxan followed by acetic-alcohol fixation × 720.

of alloxan for 1 hour at room temperature followed by treatment in coumarin at a temperature of 16—20° C. for two hours.

Well-spread metaphase plates were obtained after this procedure. In no case fragments were observed. In order to intensify the staining some of the root tips were heated for a few seconds in the acid-dye mixture. Such heating during a certain limited period no doubt gave a clearer general picture of the chromosome structure, but unexpectedly revealed the occurrence of numerous fragments in practically all division stages in the preparations. An investigation of the exact time of heating required to cause fragmentation thus became necessary. The experiments involved heating for 10, 20, 30 and 40 seconds. In every case the root

tips had to be kept in the dye-acid mixture for 5—10 minutes after heating in order to insure intense staining.

In nuclei heated for 20 seconds erosion throughout the length of the chromosomes became manifest, while heating for 30 seconds produced considerable fragmentation (Figs. 1—4, 7—11). Erosion of chromosome segments preceding fragmentation is at random. Further increase in



Figs. 7—12. Fragmentation in Allium cepa as in Figs. 1—6, 7—10 corresponding to 1—4, and 11 and 12 to 5 and 6. × 720.

the time of heating resulted in absolute disappearance of the stainable material from the chromosomes. This loss of stainability might possibly be caused by complete detachment of the nucleic acid from the chromosomal nucleoproteins.

The time of heating necessary to produce fragments depends upon the time of pretreatment. After application of 0.2% coumarin or 0.002 M oxiquinoline solutions for 3 hours at least 40 seconds of heating are necessary, but after pretreatment in these chemicals for 4 or more hours 30 seconds are sufficient to induce fragmentation.

Fragments are much more numerous in nuclei pretreated with phenols and alloxan than in those pretreated with coumarin or oxyquinoline.

Control experiments to check the role of different ingredients and steps of orcein staining.

a) Root tips were first stained in Feulgen solution instead of orcein. No fragments were observed.

b) As the Feulgen technique involves hydrolysis in N/HCl at only 60° C. it was thought that possibly stronger heating might be the main factor in producing fragmentation. Hydrolysis at 60° C. was therefore replaced by heating in N/HCl over a flame for 30 seconds. These root tips too, though showing a positive Feulgen reaction, yielded no fragments, thus proving the importance of orcein for the result.

c) In order to check the possible role of acetic acid in which orcein is dissolved, Feulgen preparations were made after heating in a mixture of equal parts of 45% acetic acid and N/HCl. No fragments were found in such preparations. In our opinion this is conclusive evidence of the essential role of orcein molecules under heat conditions in causing fragmentation of chromosomes.

d) Pretreatment in the mentioned chemicals has been found to be an absolute necessity for producing fragmentation by orcein. Without pretreatment heating in the acid-dye mixture results only in chromosome clumping.

e) Attempts have been made to decide whether the fragment producing property is an attribute of orcein alone or shared by other basic dyes. In aceto-carmine preparations no fragments could be registered. Tests with aceto-lackmoid were not conclusive, possibly because of the poor quality of the reagent used.

So far as our experience goes, it seems that the fragments arise at random, i. e. the breakages are not localised. Root tips which had been fixed with acetic-alcohol after the pretreatment in one of the chemicals stated also indicate the presence of fragments in all division stages (Figs. 5, 6, 11, 12). Investigations are now in progress to see whether in these preparations involving posttreatment in acetic-alcohol the breakages are localised.

Discussion.

The results obtained from the reported sets of experiments reveal that fragmentation by orcein in pretreated materials is brought about in a way different from the chemicals so far studied.

None of the procedures involving the Feulgen reaction, heating for 30 seconds in N/HCl or in acetic-hydrochloric acid mixture, followed by fuchsin sulphurous acid staining cause chromosome fragmentation. It can confidently be claimed that the induction of breakage is a property of the orcein molecule itself, and that heating is a necessary prerequisite for its action.

The results prove further that fragmentation cannot be caused by orcein without any pretreatment. The only observable offect in this case is a clumping of chromosomes. A pretreatment in alloxan or one of the other chemicals is, therefore, indispensable for the demonstration of the breakage-inducing property of orcein.

An explanation of the combined effects of pretreatment and orcein staining can only be given in quite a hypothetical form. In our opinion pretreatment with one of the listed chemicals causes certain changes in the nucleic acid of the chromosomes. This change possibly consists in a variable degree of depolymerisation. The presence of stickiness is an indication of the changed physical state of DNA. Simultaneously certain other chemical linkages are presumably offected. It seems not unreasonnable to assume that the linkage between DNA and protein is loosened. The depolymerised molecules of nucleic acid thus remain linked with the proteins in a labile state susceptible to removal after any radical alteration of the surrounding medium. The change in the physical state of the DNA is not uniform throughout the entire length of the chromosomes. Therefore, different segments show varying degrees of reaction to the chemicals, some retaining more nucleic acid than others which, after heat treatment, appear as eroded segments. Heating apparently leads to the complete removal of DNA from those regions where the protein linkage is extremely labile. If the protein thread after removal of the DNA is further exposed to the action of orcein, it will finally be broken whereby the fragments arise. This suggestion, though a tentative one, takes into consideration all the factors and their variables involved in the procedure.

The property of breakage must reside in the chemical structure of the orcein molecule. A thorough study of related substances belonging to the orcein group may yield information on the radicals and their configuration which are responsible for the cytological effect.

The present report gives evidence of the damaging effects of orcein staining in the study of chemical effects on chromosomes. Prolonged heating causing breakages may lead to erroneous conclusions as to the activity of the tested chemicals. Since, however, under no conditions of standard Feulgen staining chromosome breaks have been observed, this method may safely be recommended for such studies as it reduces errors in observation and interpretation.

Summary.

Plant materials of both dicotyledonous and monocotyledonous groups were subjected to treatment with alloxan followed by coumarin, phenols, oxyquinoline etc. for 3 to 4 hours.

Root tips were then heated in an orcein-acid mixture for 10 to 40 seconds and studied after mounting in 1% orcein. Clear metaphase plates

were obtained after 10 seconds of heating, whereas after 20 and 30 seconds considerable erosion and fragmentation respectively were recorded. Complete loss of stainability resulted after heating for 40 seconds.

Fragmentation is caused only by orcein under heat conditions. This is demonstrated by control series checking all variables.

Pretreatment with the chemicals stated is of absolute necessity for fragmentation. It is suggested that this pretreatment causes depolymerisation of DNA and lability of nucleoprotein linkage at certain segments from which the DNA becomes detached during subsequent heating.

Of all the chemicals tested for pretreatment effects, marked positive results were obtained with alloxan (1 hour) followed by coumarin (2 hours at 16—20° C.). Phenols also gave positive results. The other chemicals tried need slightly longer time of treatment for fragment induction.

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DESOXYRIBOSE NUCLEIC ACID (DNA) CONTENT AND SIZE OF RAT LIVER NUCLEI DURING THIOAC ETAMIDE INTOXICATION AND RECOVERY*.

By

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With 20 figures in the text.

(Eingegangen am 11. Februar 1955.)

Indroduction.

Increasing numbers of photometric DNA (Feulgen) analyses of individual nuclei in a variety of dividing and non-dividing tissues have demonstrated that, in accordance with expectations from the DNA constancy hypothesis, alterations in pattern of nuclear DNA content parallel changes in chromosome content (for review see SWIFT 1953a; TAYLOR and MCMASTER 1954). That physiological and pathological changes do not alter this relation between DNA and chromosomes has been demonstrated by DNA absorption studies on individual interphase nuclei during such changes. For example in livers of protein-depleted rats the increases in DNA are associated with shifts in frequencies from the lower to the higher polyploid classes with no change in the means of the classes (LECOMTE and DE SMUL 1952; KLEINFELD 1953). In tumors increased nuclear DNA content is associated with heteroploidy and/or mitosis (LEUCHTENBERGER, KLEIN and Klein 1952; CARNES, WEISSMANN and GOLDBERG 1952; BADER 1953a, b; PETRAKIS 1953; PETRAKIS and FOLSTAD 1954; LEUCHTENBERGER, LEUCHTENBERGER and DAVIS 1954). Combined karyometric and photometric analyses on identical nuclei show that such differences in nuclear DNA content are paralleled by proportionate differences in nuclear volume as for example in the induction of liver ploidy classes by hypophyseal growth hormone in pituitary dwarf mice (LEUCHTENBERGER and HELWEG-LARSEN 1954).

That such parallelism exists between volume and DNA content during thioacetamide (TA) induced changes and recovery is demonstrated by the combined photometric and karyometric analyses presented herein. This drug (CH_3CSNH_2) when continuously administered

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orally to rats in small doses produces in their liver parenchyma cells a series of profound cytological alterations. Of these, fully described elsewhere (RATHER 1951), the most striking are loss of cytoplasmic basophilia and marked nucleolar and nuclear enlargement. Karyometric analyses showed that the last occurred in powers of two suggesting an effect on ploidy (RATHER 1951). Since it is known that under certain conditions rhythmic increases in nuclear volume can accompany changes in protein with no change in amount of DNA (SCHRADER and LEUCHTENBERGER 1950; ALFERT 1950; ALFERT and BERN 1954), karyometric and photometric DNA (Feulgen) analyses were conducted by CARNES, BERNSTEIN and RATHER (1953) to determine the factor responsible for the nuclear enlargement in TA intoxication. The results showed that the size increases were paralleled by proportionate increases in DNA content pointing to a possible induction by TA of abnormal degrees of polyploidy. These workers did not attempt to measure the DNA content in the nuclei of recovering livers described by RATHER (1951).

The present study was undertaken, using higher concentrations given for longer periods, with a view to answering the following questions: 1. Are the greatly increased nuclear volumes in the more severe TA effects paralleled by proportional increases in nuclear DNA content; and if so do the latter follow a ploidy pattern in accordance with the DNA constancy hypothesis? 2. Are these more severe effects at all reversible; and if so is the diminution in nuclear size paralleled by proportional reductions in nuclear DNA content?

Material and Methods.

All experiments were carried out on $2^1/_2$ to 3 month old male albino rats of the Stanford colony, a highly inbred stock which was derived from the Slonaker strain. There were two experimental series: 1. to test the effect of oral TA administration; and 2. to test the reversal of the TA effect after withdrawal of the drug. In the first the rats were fed concentrations of from 30 to 40 mgs. TA per 100 mgs. of the regular stock diet for periods ranging from 3 to $7^1/_2$ months. Food consumption was determined at weekly intervals and compared to that of the controls. There was no significant difference between the two. Rough calculation indicated that in the 30 and 40 mg.-% TA diets the daily intake of TA amounted to about 40 mgs./rat/day and 60 mgs./rat/day respectively. The relatively high death rate of the animals in this series was shown by autopsy to be due to pulmonary infection. In the reversal series the rats were maintained on TA for approximately $7^1/_2$ months and then returned to the regular diet for $2^1/_2$ months. All rats were allowed full access to their diets and to drinking water at all times. Initial and terminal body weights as well as weekly gains and losses were recorded. At the desired intervals the experimental animals and controls of the same sex and approximately the same age were sacrificed by aortic exsanguination under ether anesthesia. The livers were promptly and rapidly weighed to the nearest centigram and gross pathological changes noted. After removal of representative slices for histological study the remainder of the organ was used to prepare nuclear suspensions according to the

method of RIS and MIRSKY (1949a, b) and CARNES, BERNSTEIN and RATHER (1953) as follows: after mincing, the liver was pressed through a 50 mesh copper wire screen into excess 2% sucrose kept at ice bath temperature. After two minutes enough neutral formalin was added to produce a concentration of 10% formalin in which the isolated nuclei were fixed at room temperature for one hour or longer. The fixed suspension was then centrifuged at 1800 rpm for 5 minutes, washed with several changes of distilled water and stored in 70% ethanol. Before staining the

Table 1. *Comparison of mean amounts of DNA (microspectrophometric) and mean volumes of the same individual nuclei in the liver ploidy classes of control rats of different ages.*

	Mean nuclear DNA content of classes expressed in arbitrary units				Mean nuclear volumes (segregated by their DNA content) expressed in cubic microns. range = range in size in cubic microns % differ. = difference in % between the smallest and largest nucleus in the class			
		Rat-No. 53–R–95 Age 4½ mos.	Rat-No. 53–R–91 Age 9 mos.	Rat-No. 53–R–361 Age 14 mos.		Rat-No. 53–R–95 Age 4½ mos.	Rat-No. 53–R–91 Age 9 mos.	Rat-No. 53–R–361 Age 14 mos.
Class I (2 n)	No. of meas.	16	12	none	No. of meas.	16	12	none
					range	38–109	31–109	
					% diff.	65%	71%	
	mean	.960	.940		mean	75.25	78.41	
	st. err.	±.037	±.038		st. err.	±5.57	±6.83	
Class II (4 n)	No. of meas.	73	67	19	No. of meas.	73	67	19
					range	119–210	139–260	195–320
					% diff.	43%	46%	39%
	mean	1.99	1.97	1.90	mean	185.06	190.22	267.52
	st. err.	±.029	±.022	±.040	st. err	±2.34	±3.61	±8.06
Class III (8 n)	No. of meas.	11	19	60	No. of meas.	11	19	60
					range	294–404	297–477	380–761
					% diff.	27%	37%	50%
	mean	3.95	3.87	3.96	mean	339.70	373.00	544.60
	st. err.	±.013	±.097	±.046	st. err.	±10.62	±9.92	±10.62
Class IV (16 n)	No. of meas.	none	2	21	No. of meas.	none	2	21
					range		539–800	726–1446
					% diff.		32%	49%
	mean		7.80	7.76	mean		669.5	991.43
	st. err.		—	±.122	st. err.		—	±34.33

nuclei were extracted with 95% ethanol. Suspensions of TA and control nuclei were stained simultaneously with Feulgen according to STOWELL (1945) under carefully regulated conditions and brought into 1.54 index of refraction Shillaber oil in which they were mounted. To ensure the presence on the slides of representative proportions of the nuclear classes present in each liver the suspensions were thoroughly agitated just prior to mounting. The slices for histological study were fixed in Bouin's, Carnoy's and formalin, embedded in paraffin and sectioned at 6 microns. The stains used were hematoxylin and eosin, Feulgen and Van Gieson's.

Microspectrophotometric measurement of the DNA (Feulgen) content in individual nuclei of the suspensions was done with an instrument such as was described by POLLISTER (1952b). The measuring technique and method of calculating the

relative amount of DNA per nucleus were carried out according to the methods of POLLISTER and RIS (1947). In this work all measurements were made with a Leitz apochrom. oil-immersion 90 × objective, N.A. 1.32 used in conjunction with a Leitz 8 × periplan ocular. Diameters of spherical and major and minor axes of slightly prolate nuclei were measured to an accuracy of 0.18 microns with a filar ocular micrometer, as were also the dimensions of large nucleoli and intranuclear inclusions. In the controls and milder TA effects 100 random nuclei were measured. But in the more severe TA effects a certain amount of selection was unavoidable, since only those nuclei could be measured in which the size, number

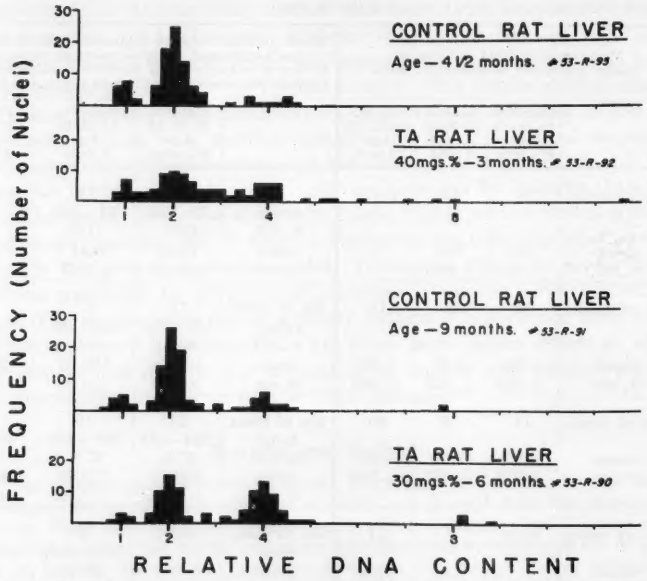

Fig. 1. Normal liver. Frequency distribution of DNA amounts, in arbitrary units, in 100 individual random nuclei from each of three normal rat livers of different ages.

and distribution of nucleoli and intranuclear inclusions were such as not to affect significantly the light path or volume. Any possible deviations in staining were corrected for by a factor relating the results of the unknown experimentals to the standard diploid nucleus.

Observations and Results.

I. Normal liver.

The results of the DNA (Feulgen) measurements on 100 random liver parenchyma nuclei from control rats of the three different age groups used in this experiment ($4^1/_2$, 9 and 14 months) show the familiar

well demarcated ploidy classes of the adult rodent liver with absence of intermediates and upward shifts of ploidy levels with age (Fig. 1 and Table 1). The results of the volume measurements on these same three sets of nuclei (Figs. 2, 3 and Table 1) show the equally familiar rough parallelism between size and DNA content of the ploidy

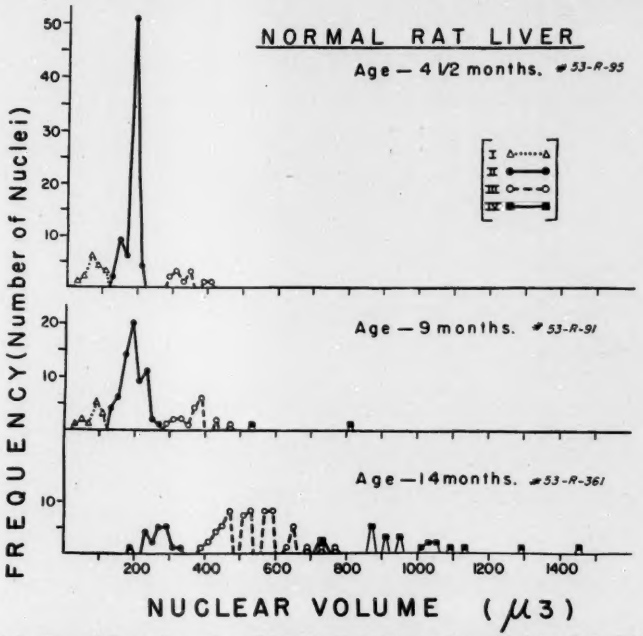

Fig. 2. Normal liver. Frequency distributions of volumes (segregated by their DNA content) of the same three sets of 100 normal nuclei as in Fig. 1.

classes. The volume data show an additional feature; namely, an increase with age in the mean nuclear volume of each ploidy class, the volumes being segregated by their DNA content. This finding supports the previous cytological demonstrations of such an increase (see discussion). To illustrate, the mean of class III (Table 1) increases from 339.7 μ^3 at $4^1/_2$ months to 377 μ^3 at 9 months (a 10% increase) and jumps to 544 μ^3 at 14 months with a 45% increase. This age change is demonstrated in graphs of amounts of DNA per nuclear volume (Fig. 3) by the upward movement along the volume axis of the means of the groups of plotted points (Fig. 4). This results in an increased slope of

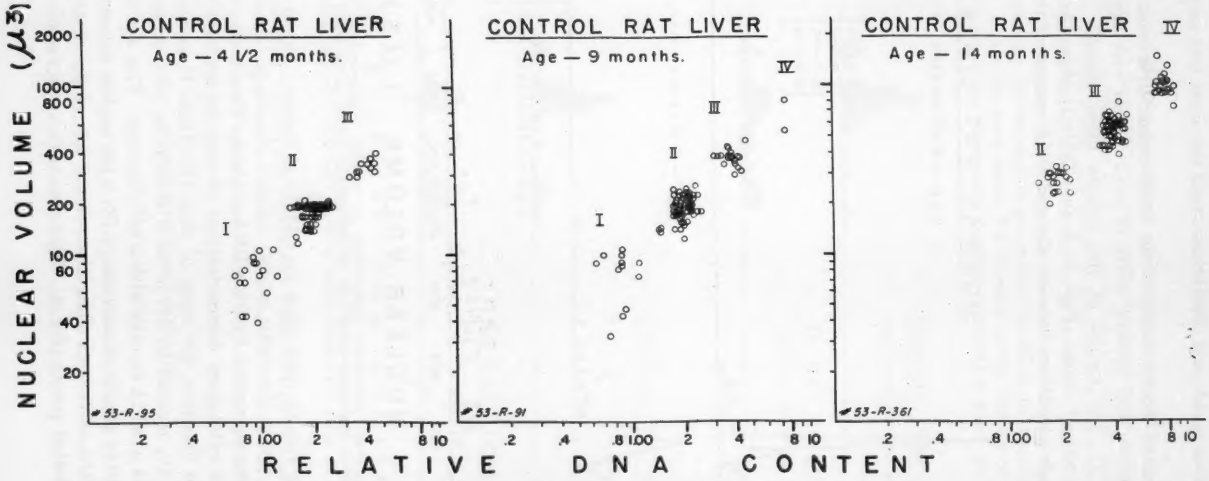

Fig. 3. Normal liver. Plots of logs of amounts of DNA per nucleus against logs of nuclear volume of the same three sets of normal nuclei as in Figs. 1 and 2.

the regression line of volume on DNA content. The volume frequency distributions (Fig. 2) reflect this size increase by 1. a progressive shift to the right in position of all classes and 2. a lateral extension of the upper class limits indicating an increased size range within each class. These changes are more marked in the higher classes. Thus in class III (Table 1) it is seen that at $4^1/_2$ months the volumes lie between

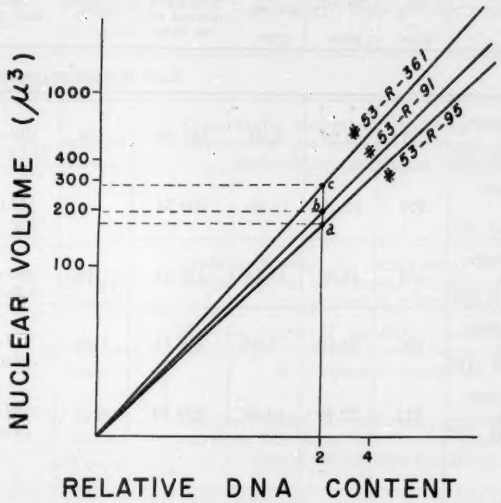

Fig. 4. Comparison of regression lines of the graphs in Fig. 3 to show the increase of slope with age. The upward shift of the mean of class II (plotted points) is illustrated.

294—404 μ^3 with a 27% difference between extremes, at 9 months between 297—477 μ^3 with a 37% difference and at 14 months between 380—761 μ^3 with a 50% difference.

Histologically these differences in size stemming from the combined effect of polyploidy and age can be seen in Figs. 5a and b.

II. Thioacetamide livers. Short and long term experiments.

A. Pathology.

The severity of the pathological changes induced in rat livers by TA, the so-called TA effect, varies with the concentration of the drug in the diet and the length of the administration period. These changes are briefly as follows (for a detailed description see Rather 1951; Ambrose, de Eds and Rather 1949): grossly the livers are enlarged

and uniformly nodular. Microscopically there is hyperplasia of bile ducts, diffuse fibrosis and enlargement of the parenchyma cells. These

Table 2. *Showing body weights, liver weights (actual and predicted), mitotic index, microscopic pathology of all rats on TA and*

	Body wt. gms.	Actual liv. wt. gms.	Predicted liv. wt. gms.	Liv. wt. in % using predicted normal wt. as base	Mitotic index	Age at kill in days and months
Rats on various concentrations						
Short term TA No. 53–R–92	217	13.29	8,24	161.28	.50	128 days (4.12 mos.)
Control No. 53–R–95	370	12.19	11.66	109.54	—	135 days (4.35 mos.)
Short term TA No. 53–R–90	351	18.37	11.19	119.48	.10	260 days (8.38 mos.)
Long term TA No. 53–R–178	300	21.19	9.99	212.11	1.10	308 days (9 mos.)
Long term TA No. 53–R–177	317	23.40	10.38	226.39	2.10	308 days (9 mos.)
Control No. 53–R–91	340	10.80	10.93	98.81	—	272 days (8.77 som.)
Rats recovering from						
Recovering No. 53–R–350	400	23.51	12.42	189.3	.50	386 days (13 mos.)
Recovering No. 53–R–360	410	21.09	12.68	166.3	.50	390 days (13 mos.)
Recovering No. 53–R–351	435	19.00	13.34	142.4%	.20	390 days (13 mos.)
Control No. 53–R–361	425	12.28	13.07	93.95	—	440 days (14.19 mos.)

show increased nuclear and nucleolar volumes with disappearance of cytoplasmic basophilia. They also show varying amounts of mitotic

age at kill, concentrations of TA used, length of administration periods and gross and after withdrawal of TA and their controls.

Conc. of TA in mgs per 100 mgs. food	Length of treatment in days and months	Pathology. Gross and Microscopic.
of TA and their controls.		
40 mgs.	88 days (2.9 mos.)	Considerable amount of nodularity moderate proliferation of bile ducts much fibrosis, almost complete loss of cytoplasmic basophilia.
—		
30 mgs.	192 days (6 mos.)	Little nodularity, no proliferation of bile ducts, limited amount of fibrosis, cytoplasmic basophilia still present in many cells.
30 mgs.	for 192 days (6 mos.)	Extreme degree of nodularity, excessive proliferation of bile ducts and fibrous tissue, complete loss of cytoplasmic basophilia.
40 mgs.	for 43 days (1.4 mos.)	Extreme degree of nodularity, excessive proliferation of bile ducts and fibrous tissue, complete loss of cytoplasmic basophilia.
total	235 days (7.4 mos.)	
thioacetamide intoxication.		
30 mgs.	for 192 days	Mildly nodular, fibrosis and hyperplasia of bile ducts still present, many paren. cells still enlarged $\bar{c}$ acidophilic cytopl., moderate no. of small cells $\bar{c}$ normal cytopl. basoph.
40 mgs.	for 43 days	Nodularity confined to left lobe, fibrosis and bile duct proliferation minimal as compared to treated rats, small cells numerous $\bar{c}$ norm. amts. of cytopl. basoph.
total	235 days (7.83 mos.)	
off TA	77 days (2.5 mos.)	No nodularity, surface mottled, fibrosis and bile ducts not noticeable, small cells extremely numerous, normal amts. of cytopl. basoph. in almost all cells.

activity which in the normal adult rat liver is virtually non-existtent as shown by the mitotic index of approximately 0.002 (BRUES and

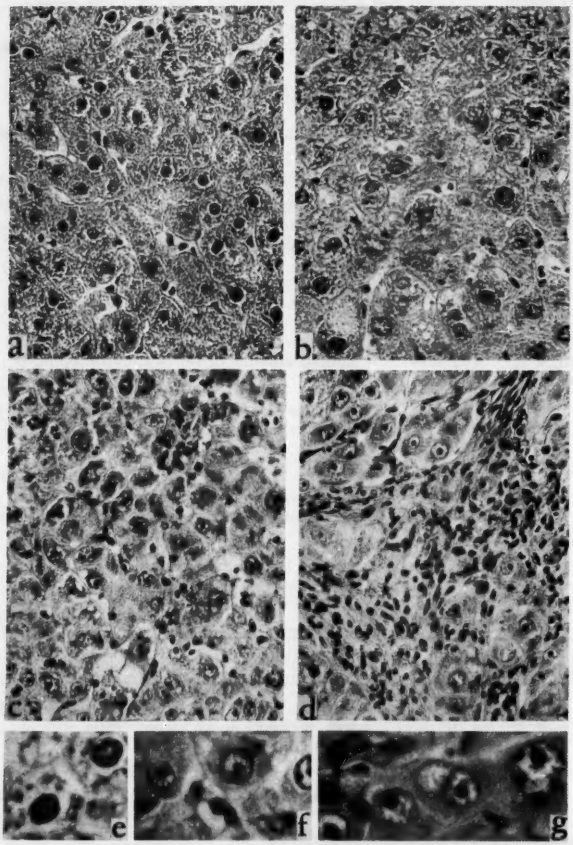

Fig. 5a—g. Photographs of normal and short term TA rat livers. a Normal rat liver, age approximately 9 months. No. 53—R—91. b Normal rat liver, age approximately 14 months. No. 53—R—361. c TA rat liver — 30 mgs.-% TA for 6 months No. 53—R—90. d TA rat liver — 40 mgs.-% TA for 3 months. No. 53—R—92. e Normal parenchyma cells of 9 month rat liver. Note cytoplasmic basophilia and small nuclei and nucleoli. f TA rat liver parenchyma cells of No. 53—R—90 showing relatively slightly enlarged nuclei and nucleoli and loss of basophilia. g TA rat liver parenchyma cells of No. 53—R—92 showing very much enlarged nuclei and nucleoli with complete loss of basophilia. Magnification of a, b, c, d 220 ×. Magnification of e, f, g 440 ×. All tissues fixed in Bouin and stained with hematoxylin and eosin.

MARBLE 1937). A brief description of the pathological changes is indicated in Table 2 as are also the mitotic indices, body weights, liver weights [expressed as per cent using the predicted normal values

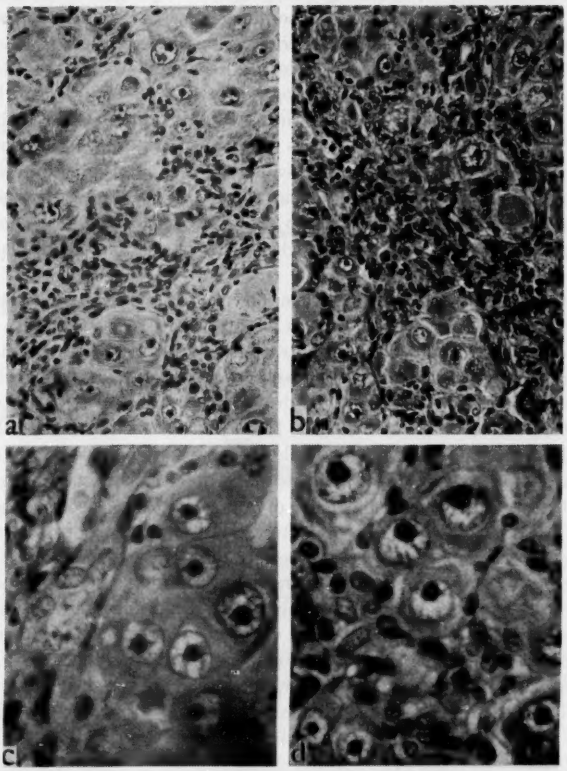

Fig. 6a—d. Photographs of long term TA rat livers. 30—40 mgs.-% TA for 7½ months. a TA liver of No. 53—R—178 showing fibrous tissue surrounding islands of parenchyma cells. b TA liver of No. 53—R—177 showing very large single cell and islands of cells isolated by abundant fibrous tissue. c TA liver of No. 53—R—178. Note extremely large nucleoli and homogeneous cytoplasm. d TA liver of No. 53—R—177. Note extremely large nucleoli. Magnification of a, b 220 ×. Magnification of c, d 440 ×. All tissues fixed in Bouin, stained with hematoxylin and eosin.

for liver weight from the equation derived from data on the Stanford colony by ADDIS and GRAY (1950)] and exact ages in days of all treated rats and their controls. Also listed are the concentrations of TA used

and the exact duration of the treatment period in days. It is seen from the table that higher concentrations (40 mgs.-%) given for shorter periods (3 months) produce a more severe effect as in rat No. 53—R—92 than lower concentrations (30 mgs.-%) given for longer periods (6 months) as in rat No. 53—R—90. Thus the first shows a greater degree of nodularity associated with a considerable increase in liver weight and loss of body weight. The second, No. 53—R—90, shows less nodularity with a relatively smaller gain in liver weight and no reduction in body weight. These differences are confirmed by the microscopic findings. Thus No. 53—R—92 (Fig. 5d) shows a greater disruption of the normal liver architecture (cf. Fig. 5a and c) caused by the increased fibrosis and proliferation of bile ducts (the latter not shown in Fig. 5 but see AMBROSE, DE EDS and RATHER 1949). It also shows greater cytological changes Fig. 5g) such as larger nuclei and nucleoli with greater loss of cytoplasmic basophilia (cf. Figs. 5f and e).

Extension of the treatment period to $7^1/_2$ months produces an exaggeration of the above effects as in rats Nos. 53—R—178 and —177 (see Table 2 for exact TA concentrations and lengths of treatment). Although these two rats received identical treatment, due to individual differences in susceptibility to the drug, No. 53—R—177 shows a more severe TA effect than No. 53—R—178. Neither of these rats shows excessive loss in body weight (Table 2) but their livers are relatively heavier than those of Nos. 53—R—92 and —90. The liver pathology consists grossly of an extreme degree of nodularity associated microscopically (Figs. 6a and b) with complete disruption of the normal orderly architecture of the organ by the excessive proliferation of fibrous tissue. This material which is scattered irregularly throughout the organ is seen to isolate the enlarged parenchyma cells either individually or in small groups. These cells display extremely large nuclei and nucleoli and abundant acidophilic cytoplasm (Figs. 6c and d; cf. control Fig. 5c). Concomitant with this general disorganization mitotic activity is markedly increased in all classes of parenchyma cells. This is roughly reflected in the elevated mitotic indices (Table 2).

B. Short term experiments. 3—6 months treatment.

1. Photometric. DNA frequency distributions.

Results of the DNA measurements in the short term experiments are shown in Fig. 7 and Table 3. The changes in DNA distribution patterns shown by TA nuclei as compared to homologous controls (Fig. 7) are 1. increased frequencies of the higher ploidy classes and 2. appearance between ploidy modal peaks of many intermediate values presumably reflecting stages in DNA synthesis. The number of these agrees roughly

with the mitotic index (Table 2). The degree to which the above changes are present depends upon the severity of the TA effect. Thus the pattern in the DNA frequency plot of the more severely affected rat No. 53—R—92 (Fig. 7) shows a greater divergence from that of its control than is the case in the milder effect of No. 53—R—90. Specifically, the first shows many more intermediate values one of which is extremely high and is

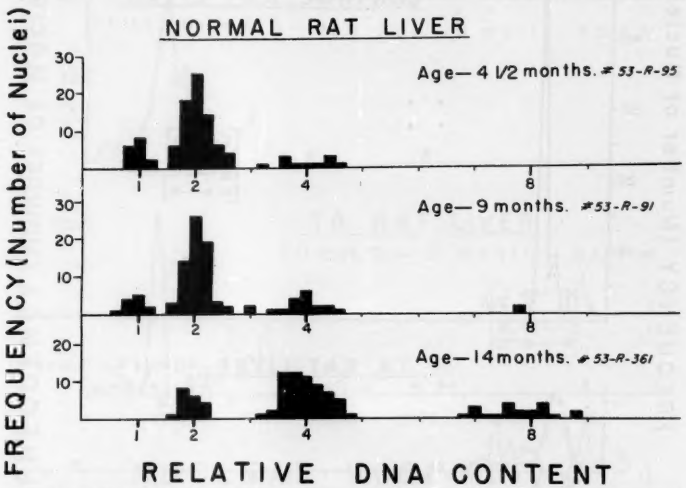

Fig. 7. Short term TA livers and homologous controls. Frequency distribution of amounts of DNA, in arbitrary units in 100 individual random nuclei from the two short term rats and their homologous controls.

seen above class IV (16n). The wide lateral spread of these values between ploidy modal peaks brings about a loss of ploidy class discreteness. This rat, No. 53—R—92, also shows a greater shift of frequencies to the right to include an extra class (16n) not present in the control. It is to be noted that this additional class is one ploidy degree above the highest in the control (8n). The second rat, No. 53—R—90, shows fewer intermediates and the upward shift is contained within control limits. In Table 3 where the means of the DNA classes are shown with their standard errors it is seen that despite the upward shifts in frequencies the TA class means remain the same as in the controls. With regard to the intermediate values, I have arbitrarily divided them into two categories; 'true' and marginal. The first are those occurring outside a distance on the abscissa equivalent to 3 times the standard deviation on either side of the mean in each normal ploidy group. They are the

values listed in Table 3 as Intermediates I, II, III etc. and shown in solid black in the graphs. The second, or marginal values, are those occurring within the extremes of this distance but in numbers in excess of expectations for a normal distribution curve. It is these marginals

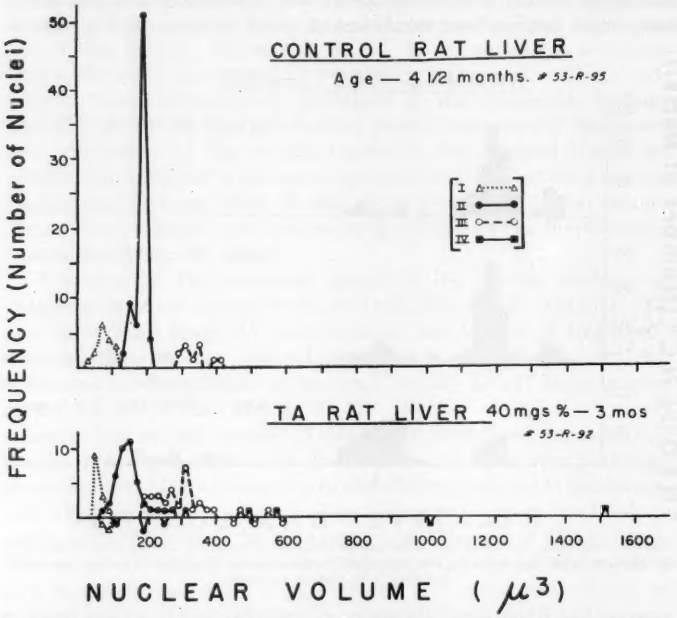

Fig. 8. Short term TA liver and homologous control. Frequency distribution of volumes (segregated by their DNA content) of the same two sets of nuclei from TA rat No. 53—R—92 and its control No. 53—R—95 as Fig. 7. 'True' intermediates indicated below the abscissa.

which are mainly responsible for the broad confluence of the classes in the DNA frequency plots of the more severely affected rat No. 53—R—92 (Fig. 7).

2. Karyometric.

a) Relation of volume to DNA content.

The relation between volume and DNA content in the two short term TA rats as compared to their controls is shown in graphs (Figs. 10 and 11) of amounts of DNA per nuclear volume. The values are plotted as before but with 25 additional specially selected large TA nuclei (black triangles). It is seen that the TA values have a linear distribution

of slope similar to the controls. This indicates the same direct proportionality between volume and DNA content in TA nuclei as in the controls. It also shows that in the TA livers the concentration of DNA in the extremely large polyploid nuclei is on the average the same as in the small diploids. However, in comparing No. 53—R—92 to

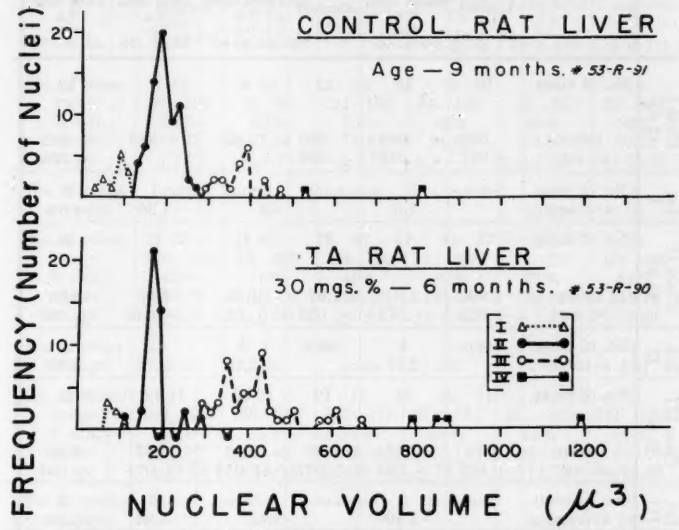

Fig. 9. Short term TA liver and homologous control. Frequency distribution of volumes (segregated by their DNA content) of the same two sets of nuclei from rat No. 53—R—90 and its control No. 53—R—91 as in Fig. 7.

No. 53—R—90 the former (Fig. 10) is seen to lack the well demarcated ploidy groups of the latter (Fig. 11) and the two controls. This is owing to the two way spread of the values from the linear relation. On the one hand is the lateral spread which is a reflection of the previously mentioned intermediate DNA values. These form a relatively narrow bridge between classes I and II connecting the upper portion of the former to the lower portion of the latter. But in the corresponding region between classes II and III they form an extremely broad band which brings about a confluence of the two classes. On the other hand is the vertical spread which stems from a greatly increased variability in size (increased size range) resulting in the overlapping of class extremes (cf. Fig. 8). In contrast to this No. 53—R—90 (Fig. 11) shows very little spread in either direction and consequently the ploidy classes stand out almost as clearly as in the control (cf. Fig. 9).

Table 3. *Comparison of mean DNA (microspectrophotometric) and mean volumes treated and*

		Mean nuclear DNA content of classes and averages of intermediates expressed in arbitrary units.					
		Control No. 53-R-95	Short term TA No. 53-R-92	Control No. 53-R-91	Short term TA No. 53-R-90	Long term TA No. 53-R-178	Long term TA No. 53-R-177
Class I (2 n)	No. of meas.	16	13	12	6	8	14
	mean	.960	.961	.940	.966	.932	.902
	st. err.	±.037	±.047	±.038	—	—	±.054
Int. I	No. of meas.	none	3	none	none	1	2
	average		1.5			1.30	1.35
Class II (4 n)	No. of meas.	73	42	67	41	27	36
	mean	1.99	2.00	1.97	1.94	2.06	2.08
	st. err.	±.029	±.071	±.022	±.031	±.056	±.050
Int. II	No. of meas.	none	4	none	3	4	1
	average		2.75		2.75	2.74	2.70
Class III (8 n)	No. of meas.	11	31	19	46	43	31
	mean	3.95	3.82	3.87	3.95	3.62	3.90
	st. err.	±.013	±.083	±.097	±.052	±.074	±.094
Int. III	No. of meas.	none	3	none	none	9	6
	average		5.40			5.84	5.55
Class IV (16 n)	No. of meas.	none	3	2	4	7	3
	mean		7.50	7.80	8.27	7.68	8.02
	st. err.		—	—	—	—	—
Int. IV	No. of meas.	none	1				2
	average		11.61	none	none	none	10.35
Class V (32 n)	No. of meas.	none	none	none	none	2	4
	mean					17.40	17.80
	st. err.					—	—
Int. V	No. of meas.						1
	average	none	none	none	none	none	22.7

b) Volume frequency distributions.

Frequency plots of the volumes (Figs. 8 and 9) of the same sets of nuclei as those in the graphs serve a dual purpose; 1. they emphasize the parallelism between volume and DNA content already shown by

of the same individual nuclei in the liver ploidy classes and intermediates of TA control rats.

Mean nuclear volumes (segregated by their DNA content) expressed in cubic microns. range = range in size in cubic microns — % differ. = difference in % between the smallest and largest nucleus in the class.

	Control No. 53-R-95	Short term TA No. 53-R-92	Control No. 53-R-91	Short term TA No. 53-R-90	Long term TA No. 53-R-178	Long term TA No. 53-R-177
No. of meas.	16	13	12	6	8	14
range	38–109	40–84	31–109	66–115	59–99	32–191
% diff.	65%	52%	71%	42%	40%	83%
mean	75.25	57.54	78.41	84.67	74.50	80.21
st. err.	±5.57	±3.28	±6.83	—	±5.03	±13.89
No. of meas.	none	3	none	none	1	2
average		85			109	68
No. of meas.	73	42	67	41	27	36
range	119–210	63–305	139–260	111–297	75–310	93–604
% diff.	43%	79%	46%	62%	75%	84%
mean	185.06	149.00	190.22	181.40	204.50	246.16
st. err.	±2.34	±7.10	±3.61	±4.45	±9.23	±19.89
No. of meas.		4		3	4	1
average	none	185	none	251	216	458
No. of meas.	11	31	19	46	43	31
range	294–404	186–541	297–477	281–661	211–561	141–1201
% diff.	27%	65%	37%	57%	62%	88%
mean	339.70	292.42	373.00	413.76	389.28	490.00
st. err.	±10.62	±14.09	±9.92	±13.19	±12.76	±46.60
No. of meas.	none	3	none	none	9	6
average		515			600	617
No. of meas.	none	3	2	4	7	3
range		493–1516	539–800	782–1180	696–1232	435–1780
% diff.		67%	32%	33%	43%	76%
mean		857.31	669.50	921.20	829.81	1225.0
st. err.		—	—	—	—	—
No. of meas.	none	1	none	none	none	2
average		1000				1733
No. of meas.	none	none	none	none	2	4
range					1910–2604	2361–3523
% diff.					26%	33%
mean					2257	2910
st. err.					—	—
No. of meas.						1
average	none	none	none	none	none	4025

the graphs (Figs. 10 and 11) and 2. they show more clearly the intraclass increases in size range over the normal as well as the overlapping of class extremes mentioned in the previous section. With respect to the first, the volume-DNA parallelism, it is seen that the frequency plots

of volumes (Figs. 8 and 9) and DNA content (Fig. 7) are remarkably alike; the clear cut DNA classes of the controls Nos. 53—R—95 and —91 (Fig. 7) show similar clear cut volume classes (Figs. 8 and 9); and the

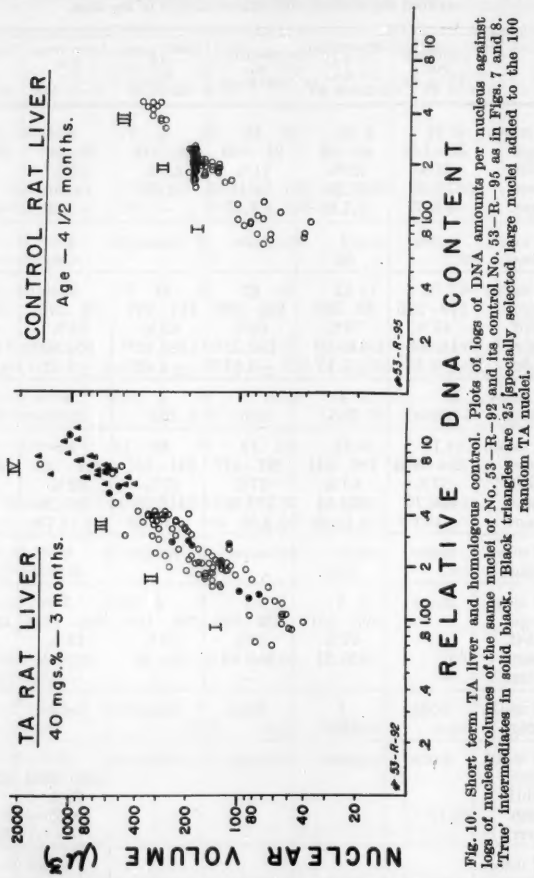

Fig. 10. Short term TA liver and homologous control. Plots of logs of DNA amounts per nucleus against logs of nuclear volumes of the same nuclei of No. 53—R—92 and its control No. 53—R—95 as in Figs. 7 and 8. 'True' intermediates in solid black. Black triangles are 25 specially selected large nuclei added to the 100 random TA nuclei.

loss of this discreteness in the DNA classes of the TA rats is paralleled by a similar flowing together of volume class boundaries. This effect, although hardly noticeable in No. 53—R—90 (Fig. 9), is quite considerable in No. 53—R—92 (Fig. 8). Also high DNA values are matched by large volumes. With respect to the second or size range increases, as compared

to controls, both TA rats show them in all classes except I. However in No. 53—R—90 they are relatively not as great as in No. 53—R—92. This is owing to the fact that in the former only the upper class boundaries

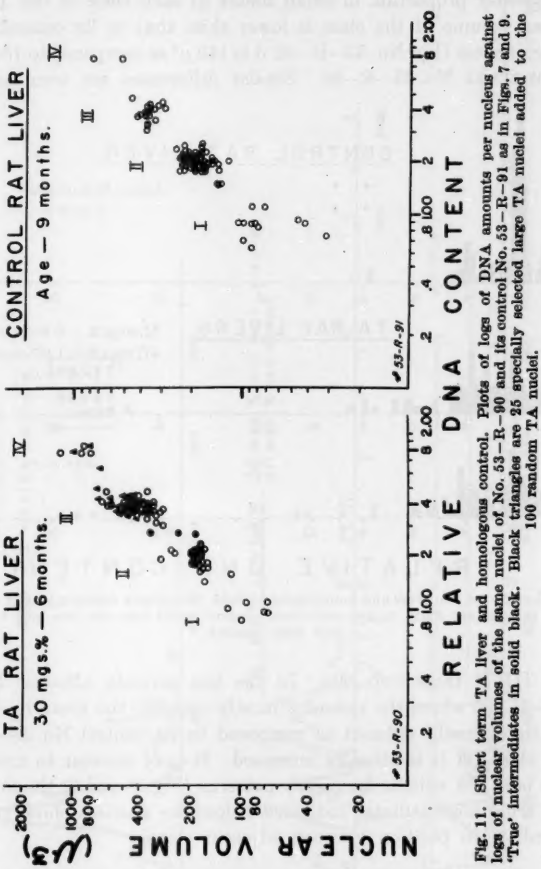

Fig. 11. Short term TA liver and homologous control. Plots of logs of DNA amounts per nucleus against logs of nuclear volumes of the same nuclei of No. 53—R—90 and its control No. 53—R—91 as in Figs. 7 and 9. 'True' intermediates in solid black. Black triangles are 25 specially selected large TA nuclei added to the 100 random TA nuclei.

exceed control limits, whereas in the latter both upper and lower boundaries are thus extended. Such a two way expansion results in the overlapping of class extremes and the lowering of the mean class volumes in No. 53—R—92 (Table 3). More specifically, class II of this rat, as is seen from the table, shows a range of from 63—305 μ^3 (a difference

between extremes of 79%). This is about $2^1/_2$ times as great as that of the corresponding class of the control, No. 53—R—95, with a range from 119—210 μ^3 (a 43% difference between extremes). However owing to the greater proportion of small nuclei in each class of the TA rat the mean volume of the class is lower than that in its control. For example, in class II of No. 53—R—92 it is 149 μ^3 as compared to 185 μ^3 in the control rat No. 53—R—95. Similar differences are seen between

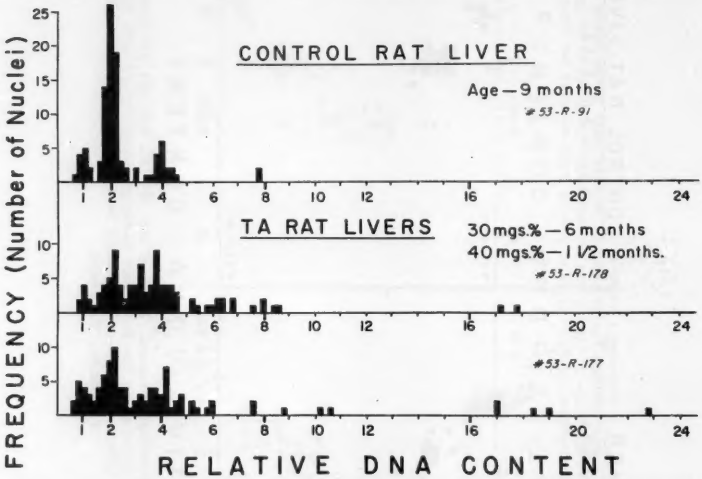

Fig. 12. Long term TA livers and homologous control. Frequency distributions of amounts of DNA, in arbitrary units, in 100 individual random nuclei from the two long term rats and their control.

classes III of these two rats. In the less severely affected TA rat No. 53—R—90 where the spread is mostly upward, the mean in class II is not significantly reduced as compared to its control No. 53—R—91 and in class III it is actually increased. It is of interest to note here that in both TA volume frequency patterns (Figs.8 and 9) the majority of the 'true' intermediates indicated below the abscissa show volumes intermediate in position between adjacent classes.

C. Long term experiments — $7^1/_2$ months treatment.

1. Photometric. DNA frequency distributions.

The degree of alteration in patterns of nuclear DNA content in rats treated for $7^1/_2$ months as compared to their control (Fig. 12) is similar to that already seen in the more severe TA effect, No. 53—R—92, of

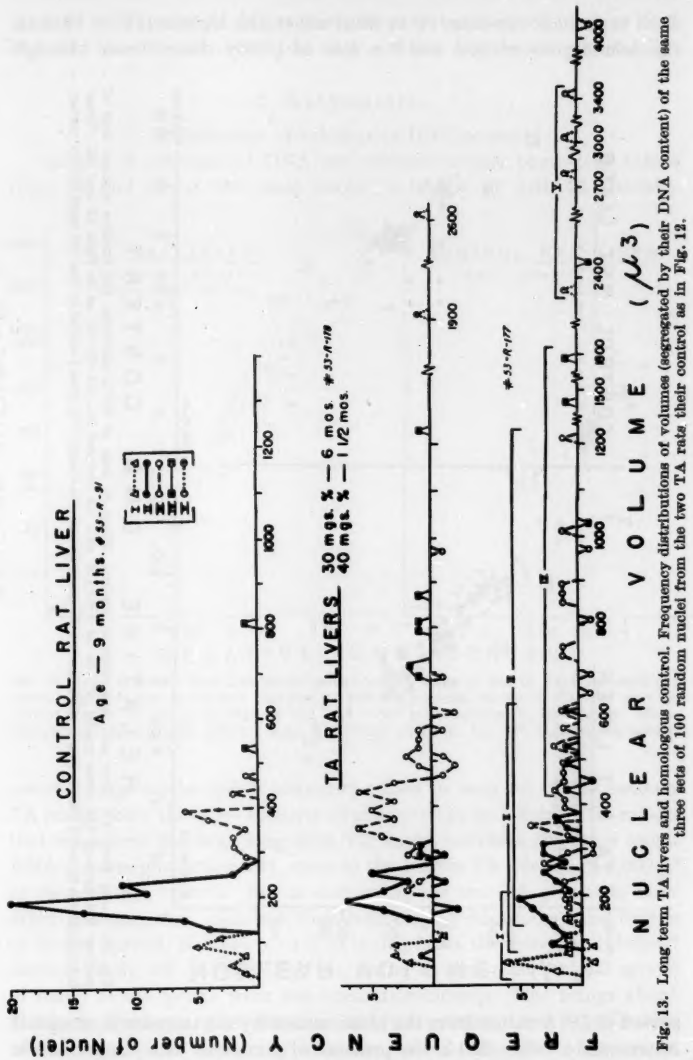

Fig. 13. Long term TA livers and homologous control. Frequency distributions of volumes (segregated by their DNA content) of the same three sets of 100 random nuclei from the two TA rats their control as in Fig. 12.

the short term experiments (cf. Fig. 7). Thus the DNA frequency plots of Nos. 53—R—178 and —177 show 1. a shift of DNA values to the

right to include one class (V or 32n) above the highest (IV or 16n) in the homologous control and 2. a loss of ploidy discreteness through

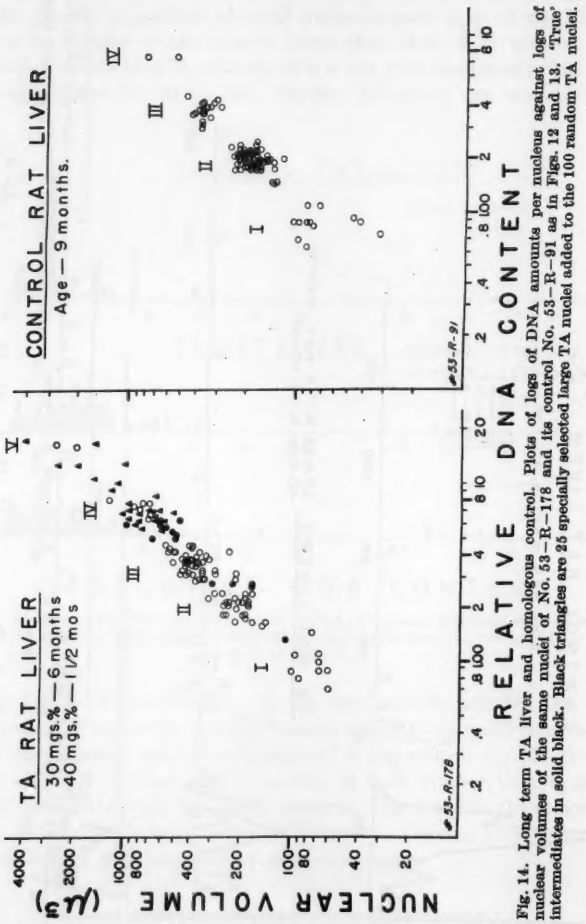

Fig. 14. Long term TA liver and homologous control. Plots of logs of DNA amounts per nucleus against logs of nuclear volumes of the same nuclei of No. 53–R–178 and its control No. 53–R–91 as in Figs. 12 and 13. 'True' intermediates in solid black. Black triangles are 25 specially selected large TA nuclei added to the 100 random TA nuclei.

spread of DNA values from the mean caused by the increase in marginal intermediate values and 3. the presence of numerous 'true' intermediate values (Table 3). Here it is seen that, as before, despite considerable shifts in class frequencies, the means of the DNA classes remain the

same as in the control. Slight increases may be observed in class II of Nos. 53—R—178 and —177 but they are not significant statistically.

2. Karyometric.

a) Relations of volumes to DNA content.

Graphs of amounts of DNA per nuclear volume plotted as before (Figs. 14 and 15) of the same nuclei as in Fig. 12 with 25 specially

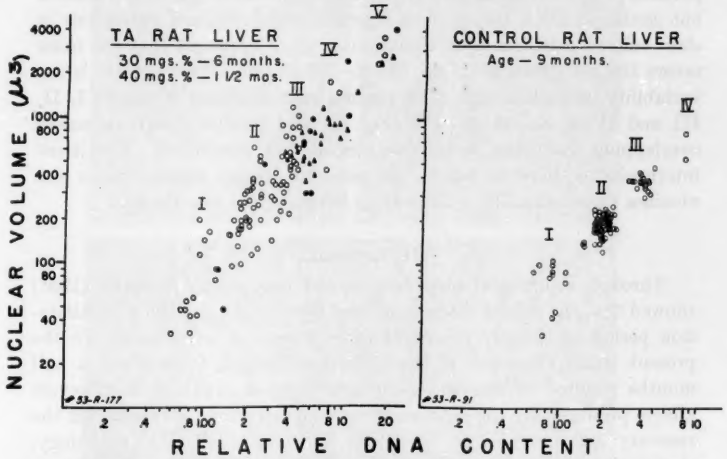

Fig. 15. Long term TA liver and homologous control. Plots of logs of DNA amounts per nucleus against logs of nuclear volumes of the same nuclei of No. 53—R—177 and its control No. 53—R—91 as in Figs. 12 and 13. 'True' intermediates in solid black. Black triangles are 25 specially selected large TA nuclei added to the 100 random TA nuclei.

selected large nuclei (black triangles) added to each set of 100 random TA nuclei show the same linearity of similar slope as before. This means that in general the large long term TA nuclei maintain the same rough DNA-volume proportionality, even to the largest TA nucleus of 4,000 μ^3 as those in the control. But in comparing the two TA graphs to each other it is seen that, although they both display about the same degree of lateral spread, No. 53—R—177 (Fig. 15) lacks the fairly well defined clusters of No. 53—R—178 (Fig. 14), owing to a marked vertical spread of many of the points from the linear relationship. This brings about extensive overlapping of the class volume extremes and is indicative of the tremendous variability in nuclear size in this liver. Because of this wide vertical spread the previously mentioned bands of intermediates extending between classes are not discernible in No. 53—R—177 (Fig. 15)

although in No. 53—R—178 (Fig. 14), where there is much less spread, they are quite clearly recognizable.

b) Volume frequency distributions.

Comparison of the DNA (Fig. 12) and volume (Fig. 13) frequency distributions (volumes segregated by their DNA content) of the same nuclei demonstrates as before the similarity between the two types of patterns; i. e. well demarcated DNA classes have clear cut volume classes, but confluent DNA classes show upward and downward extensions of class volume extremes with overlapping. It is apparent that the more severe the TA effect, as in No. 53—R—177 (Table 2), the greater is the variability in nuclear size. This reaches such extremes in classes I, II, III and IV of No. 53—R—177 (Fig. 13) and produces such extensive overlapping that class boundaries are all but eliminated. The 'true' intermediates, here as before, are seen to occupy regions below the abscissa approximately intermediate between the size classes.

III. Reversal.

Through histological observations and karyometry RATHER (1951) showed that the milder changes induced by a brief 4 day TA administration period are largely reversible after 9 days of withdrawal. In the present study extension of the withdrawal period to two and a half months resulted in almost complete reversal of even the most severe effects produced by the $7^1/_2$ month treatment period. Evidence for the recovery was provided by the three following studies: 1. pathology, showing return of comparatively normal gross and microscopic pictures, 2. karyometry, showing a reduction of nuclear volumes to normal levels and 3. photometry, showing a reduction in DNA content paralleling that in volumes.

A. Pathology. Gross and microscopic.

Three rats Nos. 53—R—350,—360 and —351 all received similar concentrations (30—40 mgs.-% of TA in the diet) (see Table 2 for exact length of treatment periods) for the same period ($7^1/_2$ months) as Nos. 53—R—178 and —177 but were taken off for $2^1/_2$ months before the kill. Owing to individual differences in reaction to the drug and rates of recovery from its effect they provide a convenient series illustrating stages in the recovery process (Table 2). Thus No. 53—R—350 is judged to be the least normal, since it shows the least gain in body weight, least reduction in liver weight and greatest amounts of nodularity still present. Using the same criteria No. 53—R—351 is considered as the most completely recovered and No. 53—R—360 as intermediate

between the two. The microscopic histology confirms this seriation. Thus No. 53—R—350 (Fig. 16a) shows a large amount of fibrous tissue and numerous enlarged nuclei while No. 53—R—351 (Fig. 16c) shows

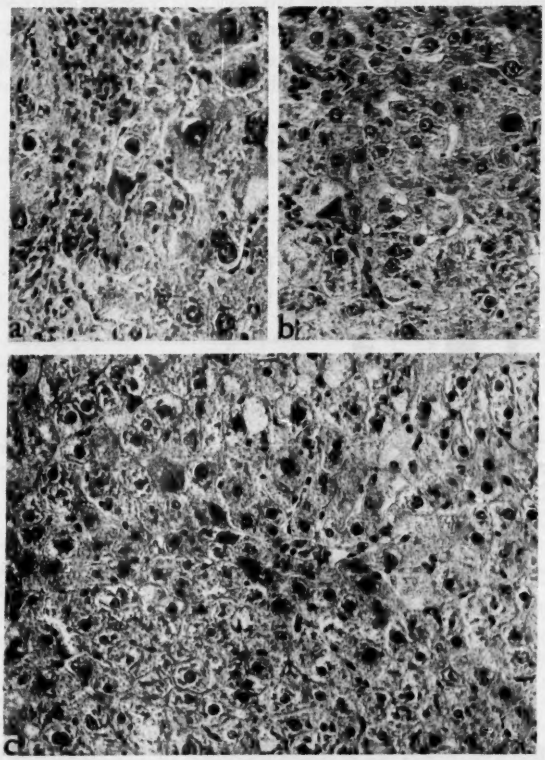

Fig. 16a—c. Reversal. Photographs of rat livers in three different stages of recovery. a No. 53—R—350 showing least recovery. Note larger nuclei, and greater amount of fibrous tissue. b No. 53—R—360 showing intermediate amount of recovery. Note fewer large nuclei and reduced amounts of fibrous tissue. c No. 53—R—351 showing almost complete recovery. Note large numbers of small cells with small nuclei and nucleoli, and small amounts of fibrous tissue. Magnification of a, b, c 220 ×. All tissues fixed in Bouin and stained with hematoxylin and eosin.

a crowding of the fibrous tissue into narrow bands (not shown here) by numerous tightly packed small diploid cells. These show normal sized nuclei, nucleoli and amounts of cytoplasmic basophilia (cf. Fig. 5a). In all these respects No. 53—R—360 is intermediate between the two

foregoing. Mitotic activity could still be observed in the sections of all three rats, although No. 53—R—351 showed less than the other two (Table 2).

B. Photometric. DNA frequency distributions.

The patterns of nuclear DNA content in the recovering livers (Fig. 17) are characterized by reversal of all the changes induced by TA described

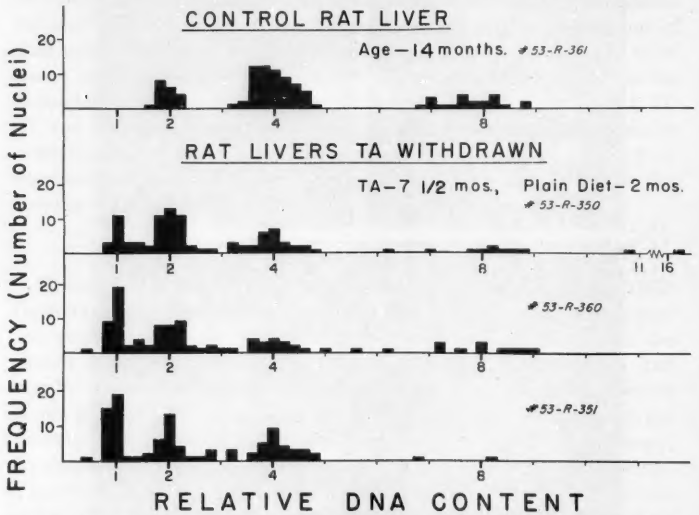

Fig. 17. Reversal. Frequency distribution of amounts of DNA in arbitrary units in 100 individual random nuclei from the three recovering rat livers shown in Fig. 16 and their homologous control.

above. Just as TA effects a shift in frequencies to the right associated with confluence of ploidy classes (Figs. 7 and 12), so recovery produces a shift to the left with decreased frequencies of the higher ploidy classes and a return of fairly normal class discreteness. As before, despite this considerable shifting in class frequencies, the means of the various DNA classes remain unaltered (Table 4). Comparing the DNA patterns of the three recovering rats Nos. 53—R—350, —360 and —351 to each other (Fig. 17) it is seen that the degree of shift to the left agrees with the seriation previously established (see Table 2). Specifically the least normal rat No. 53—R—350 shows 1. the greatest extension to the right as evinced by the presence of members of class V (32n) and of high intermediates and 2. the lowest frequencies in class I (2n). In contrast to this, the most completely recovered rat No. 53—R—351 shows 1. no

abnormally elevated DNA values and 2. the highest frequencies in class I. In all the foregoing respects No. 53—R—360 is approximately intermediate between the two. It is to be noted that the dissimilarity between the recovery patterns and that of their homologous control No. 53—R—361 is attributable to the build up in the former of the

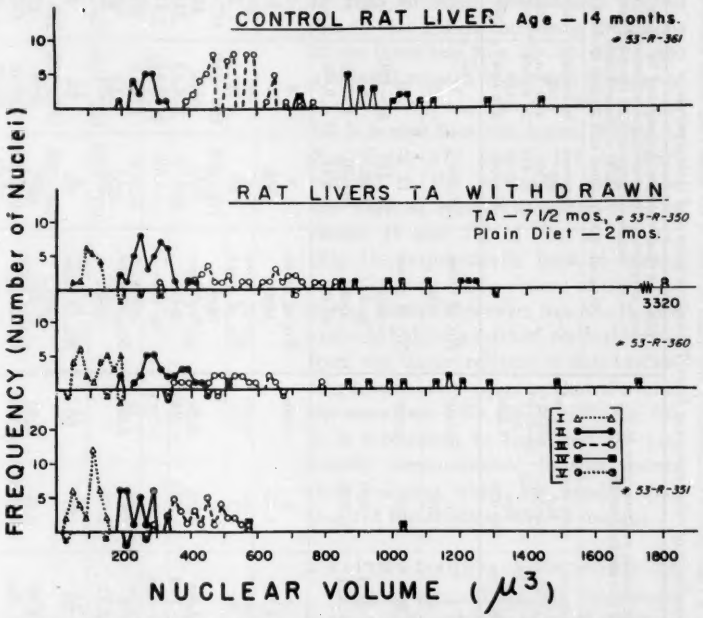

Fig. 18. Reversal. Frequency distribution of volumes (segregated by their DNA content) of the same individual nuclei from the three recovering rats and their control as Fig. 17. 'True' intermediates shown below the abscissa.

above mentioned diploid values belonging to the numerous small diploid nuclei seen throughout the sections in progressively increasing numbers proceeding from No. 53—R—350 to —351. It is the considerable mitotic activity of these which is responsible for the persistence of a higher than normal mitotic index (Table 2). A feature of the last two frequency plots so far not encountered in any of the others is the presence in each of a single haploid value (see discussion).

C. Karyometric.

1. Relation of volume to DNA content.

Graphs plotted as before of amounts of DNA per nuclear volume (Figs. 19 and 20) of the same nuclei from the recovering rats and their

Table 4. *Comparison of mean amounts of DNA (microspectrophotometric) and mean volumes of the same individual nuclei in the liver ploidy classes and intermediates of rats recovering from TA intoxication and their controls.*

	Mean nuclear DNA content of classes and averages of intermediates expressed in arbitrary units.					Mean nuclear volumes (segregated by their DNA content) and averages of intermediates expressed in cubic microns. range = range in size in cubic microns % diff. = difference in % between the smallest and largest nucleus in the class				
		Recovering Rat-No. 53–R–350 Age 386 dys.	Recovering Rat-No. 53–R–360 Age 390 dys.	Recovering Rat-No. 53–R–351 Age 390 dys.	Control Rat-No. 53–R–361 Age 14 mos.		Recovering Rat-No. 53–R–350 Age 386 dys.	Recovering Rat-No. 53–R–360 Age 390 dys.	Recovering Rat-No. 53–R–351 Age 390 dys.	Control Rat-No. 53–R–361 Age 14 mos.
Hap. (n)	No. of meas.	none	1	1	none	No. of meas.	none	1	1	none
	mean		.43	.30		size		29	23	
Class I (2 n)	No. of meas.	17	30	35	none	No. of meas.	17	30	35	none
						range	59–127	41–190	34–152	
						% diff.	53%	78%	77%	
	mean	.963	.953	.920		mean	101.30	120.03	94.97	
	st. err.	±.027	±.021	±.018		st. err.	±5.41	±9.06	±5.65	
Int. I	No. of meas.	3	4	1	none	No. of meas.	3	4	1	
	aver.	1.37	1.36	1.42		aver.	231	211	147	none
Class II (4 n)	No. of meas.	40	30	27	19	No. of meas.	40	30	27	19
						range	192–404	180–510	187–328	195–320
						% diff.	52%	64%	42%	39%
	mean	1.97	1.99	1.98	1.90	mean	283.70	314.33	243.00	267.52
	st. err.	±.036	±.044	±.044	±.040	st. err.	±7.86	±12.54	±17.80	±8.06
Int. II	No. of meas.	1	2	3	none	No. of meas.	1	2	3	none
	aver.	2.73	2.72	2.76		aver.	255	283	232	
Class III (8 n)	No. of meas.	28	20	31	60	No. of meas.	28	20	31	60
						range	309–836	356–784	295–561	380–761
						% diff.	63%	54%	47%	50%
	mean	3.92	3.92	3.98	3.96	mean	578.25	520.35	432.54	544.6
	st. err.	±.079	±.080	±.072	±.046	st. err.	±25.82	±23.91	±13.10	±10.62

Int. III	No. of meas. aver.	1 6.20	2 5.87	none	21	No. of meas. aver.	1 705	2 570	none	21
Class IV (16 n)	No. of meas.	8	11	2		No. of meas. range	8 851–1253	11 861–1737	2 573–1028	726–1446
						% diff.	32%	50%	44%	49%
	mean	7.20	7.96	7.45	7.76	mean	1070.8	1182.9	800.5	991.43
	st. err.	—	±.022	—	±.122	st. err.	—	±140.78	—	±34.33
Int. IV	No of. meas. aver.	1 10.80	none	none	none	No. of meas. size	1 1305	none	none	none
Class V (32 n)	No. of meas. amount	1 16.28	none	none	none	No. of meas. size	1 3328	none	none	none

control as in Fig. 17 show that decreases in nuclear volume parallel decreases in their DNA content. This is evidenced as before by the linear distribution of the class means with a slope similar to that of their homologous control No. 53—R—361. In comparing the graphs of the three rats Nos. 53—R—350, —360 and —351 to each other and to those of the long term experiments (Figs. 14 and 15) it is seen that the lateral spread of Nos. 53—R—178 and —177 is much reduced in the recovering rats. Also the vertical spread, so conspicuous in classes II and III of No. 53—R—177 (Fig. 15), is practically back to normal in the corresponding classes of the recovering livers. However, Nos. 53—R—360 and —351 show a marked vertical spread from the linear relation in class I reflecting a variability equal to that shown in the same class of No. 53—R—177 (Fig. 15). It is interesting to note that the previously demonstrated haploid values show volumes which are smaller than those of the smallest diploid nucleus.

2. Volume frequency distributions.

Just as in the TA nuclei the volume pattern followed the upward shift in DNA frequencies, so here the volume patterns during recovery parallel the downward shift of DNA levels. The progressive shift to the left of volume frequencies proceeding from No. 53—R—350 through —360 to —351 is shown in Fig. 18. Here it is seen that this is effected by a progressive retraction of the upper limits of each class (class I excepted). In class III for example (Table 4) it is reduced from 836 μ^3 in No. 53—R—350 to 561 μ^3 in No. 53—R—351. In addition to this, the

low levels reached by the lower class limits in Nos. 53—R—178 and —177 (Fig. 13, Table 2) are raised in the recovering rats to approximately the normal levels of the homologous control No. 53—R—361

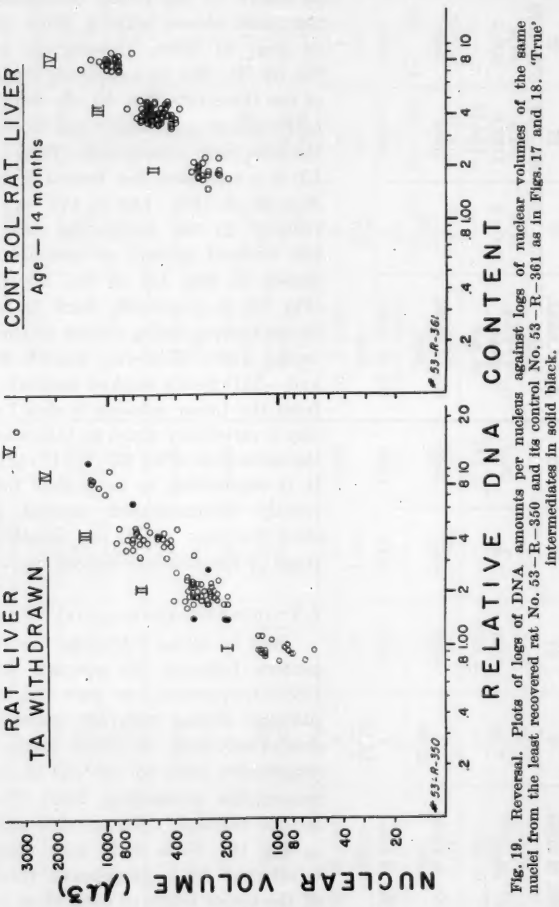

Fig. 19. Reversal. Plots of logs of DNA amounts per nucleus against logs of nuclear volumes of the same nuclei from the least recovered rat No. 53—R—350 and its control No. 53—R—361 as in Figs. 17 and 18. 'True' intermediates in solid black.

(Fig. 18). Such combined retraction of class limits reduces progressively the size range within each class. For example class III (Table 4) in No. 53—R—350 shows a difference between extremes of 63% which

is reduced to 47% in —351. These contractions of class boundaries also reduce the total pattern length in No. 53—R—351 to about half that in No. 53—R—350 and at the same time practically eliminate overlapping.

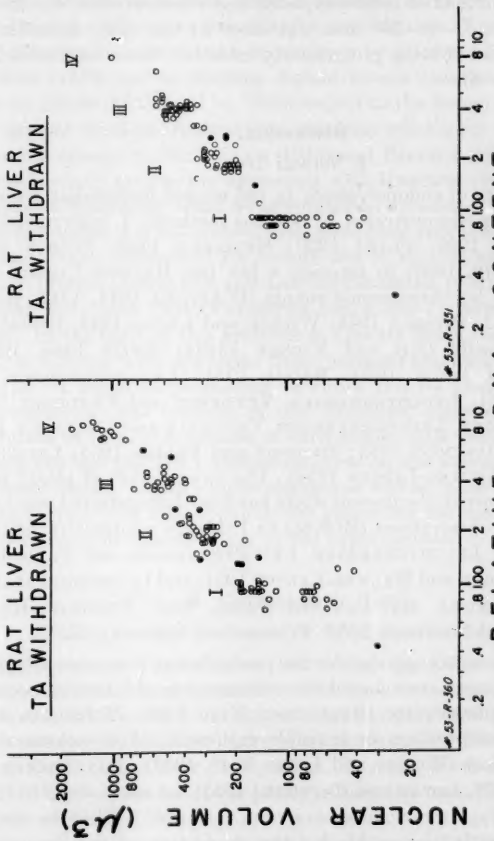

Fig. 20. Reversal. Plots of logs of DNA amounts per nucleus against logs of nuclear volumes of the same nuclei from the intermediate rat No. 53—R—360 and the most completely recovered rat No. 53—R—351 as in Figs. 17 and 18. 'True' intermediates in solid black. Haploid values — circle with dot in center.

It should be pointed out here that the end result of such combined shifts produces in No. 53—R—351 a pattern unlike that of its homologous control No. 53—R—361 but quite similar to that of the 9 month control No. 53—R—91 (Fig. 13). Thus, although both Nos. 53—R—351 and —91 show four classes, the predominant type in the recovering rat is the diploid as contrasted to the tetraploid in the control. Then, of course,

the former shows a small amount of overlapping and a few intermediates both of which are absent in the normal liver. The changes in class I in these three rats differ from those taking place in the higher ploidy classes. Thus in the two most completely recovered rats Nos. 53—R—360 and —351 (No. 53—R—350 does not show it) the upper boundary of this class instead of being progressively retracted shows an inreased expansion (Table 4).

Discussion.

I. Normal liver.

The presence of endopolyploidy in the normal mammalian liver has been repeatedly demonstrated by various methods; 1. karyometrically (JACOBJ 1925, 1935; CLARA 1930; MICHAELIS 1938; SULKIN 1943; SCHREIBER 1949, 1950) to mention a few (see HELWEG-LARSEN 1952 for review), 2. by chromosome counts (D'ANCONA 1941, 1942; BEAMS and KING 1942; BIESELE 1944; WILSON and LEDUC 1948, 1950a) and 3. photometrically (RIS and MIRSKY 1949a; SWIFT 1949, 1950a; PASTEELS and LISON 1950; NAORA 1951; LEUCHTENBERGER and SCHRADER 1951; LEUCHTENBERGER, VENDRELY and VENDRELY 1951; LEUCHTENBERGER, LEUCHTENBERGER, VENDRELY and VENDRELY 1952; FRAZER and DAVIDSON 1953; THOMSON and FRAZER 1954; LEUCHTENBERGER and HELWEG-LARSEN 1954). The upward shift of ploidy levels with age as shown in the present study has been demonstrated previously by cytological observations (MCKELLAR 1949), by photometric analyses (SWIFT 1950a; LEUCHTENBERGER, LEUCHTENBERGER and DAVIS 1954; LEUCHTENBERGER and HELWEG-LARSEN 1954) and by chemical analyses (SIBATANI, FUKUDA, MATSUDA and NAORA 1952; THOMSON, HEAGY, HUTCHISON and DAVIDSON 1953; FUKUDA and SIBATANI 1953).

The mechanisms suggested for the production of liver endopolyploidy are the following; 1. merging of the chromosomes of binucleate cells on a single metaphase plate (BEAMS and KING 1942; MCKELLAR 1949) and 2. endoreduplication or invisible replication of the chromonema during interphase (WILSON and LEDUC 1948, 1950a; FANKHAUSER and HUMPHREY 1952; LEVAN and HAUSCHKA 1953), see also LORZ (1947) and HUSKINS (1947); (for reviews see GEITLER 1941, 1953). That the etiological factor directly responsible for the production of the liver ploidy classes is the hypophyseal growth hormone was conclusively demonstrated by LEUCHTENBERGER and HELWEG-LARSEN (1954).

Since in the TA effect the relation between nuclear size and DNA content was of major import the two types of nuclear size increase in the control livers had to be taken into consideration. They are 1. rhythmic doublings of nuclear volumes paralleling those of the DNA content

of the ploidy classes and 2. progressive increase with age of the mean class volumes. With respect to the first the present results are in agreement with the previous demonstrations of a rough proportionality between volume and DNA content (number of chromosome sets) in non-dividing adult mouse tissues by SWIFT (1950a) and LEUCHTENBERGER and HELWEG-LARSEN (1954), in rat liver by CARNES, BERNSTEIN and RATHER (1953) and in dividing diploid tissues (number of chromatid sets) by SWIFT (1953a and b). With respect to the second or age increase the present findings confirm the previous cytological demonstration of such a change by MCKELLAR (1949) and BIESELE (1944). Although it was formerly assumed in agreement with HERTWIG (1935, 1939) that all volume differences could be attributed to differences in chromosome mass or number it is now known that, aside from polyploidy and mitosis, nuclear volume differences are mainly due to varying amounts of non-histone protein (SCHRADER and LEUCHTENBERGER 1950; ALFERT 1950; ALFERT and BERN 1951; POLLISTER 1952a and c; BERN and ALFERT 1954). In the light of this evidence it appears likely that the increase in class volumes with age stems from increased amounts of non-histone protein. It would be interesting to verify this by photometric Millon measurements. The presence of this additional material brings about a dilution of the DNA content of each ploidy class with age, although at any one age the DNA concentration is on the average the same in all ploidy classes. These volume changes as well as those in ploidy levels in the normal liver make obvious the reason for the use in the present study of controls of similar age as the experimentals.

II. Thioacetamide livers.

A. Interpretation of changes in DNA distribution patterns.

The questions originally set concerning the DNA content of the enlarged hepatic nuclei in the TA effect have been clearly answered as follows; 1. the increased nuclear volumes are paralleled by increases in DNA content; and 2. these take place rhythmically fitting the normal 1:2:4:8 etc. normal liver ploidy series. In other words despite upward shifts in the frequencies of the ploidy classes the means of these classes remain unchanged as compared to controls. In direct disagreement with these results are the chemical analyses on TA livers by LAIRD (1953) and THOMSON, HEAGY, HUTCHINSON and DAVIDSON (1953) who report finding no change in the average hepatic nuclear DNA content. However the discrepancies are not serious when it is considered that both analyses covered short experimental periods, the first from 4 to 28 days, and the second 7 days, whereas the shortest period for which DNA increases have been shown photometrically is 84 days (CARNES,

BERNSTEIN and RATHER 1953). Furthermore it must be remembered that chemical analyses of a cell population consisting of such diverse types as are found in the liver cannot give a reliable picture of nuclear DNA content in one of these types. This is especially true when the single type, here the parenchyma cell, is diversified by varying degrees of polyploidy and mitosis.

A question which cannot be answered as clearly as those originally set arises in connection with the DNA distribution patterns presented above: Should they be interpreted simply as 1. reflections of mitosis in a polyploid tissue as suggested by LEUCHTENBERGER, LEUCHTENBERGER and DAVIS (1954), or 2. as manifestations of an actual induced polyploidy accompanied by mitosis as suggested by BADER (1953b) and HEIZER and RATHER (1954)? In favor of the first interpretation is the close resemblance between the patterns in the more severe TA effects as in No. 53—R—92 (Fig. 7) and Nos. 53—R—177 and —178 (Fig. 12) and those found by many investigators in normal dividing tissues (SWIFT 1950a, b; ALFERT 1950; POLLISTER, SWIFT and ALFERT 1951; MOORE 1952; MCMASTER 1952; SWIFT and KLEINFELD 1953; ALFERT and SWIFT 1953; PATAU and SWIFT 1953; PETRAKIS 1953; TAYLOR and MCMASTER 1954; MARINONE 1954). Thus the two display the following features in common 1. a spread of values between ploidy modal peaks — in dividing tissues between the diploid and twice the diploid amounts; in the TA livers between all the ploidy classes, and 2. shifts of frequencies to one class above the highest in the control — in dividing tissues from 2n to 4n; in TA livers from Xn to Xn+Xn. The first of these features in now known to represent DNA synthesis (but see below) and the second stems from the temporary accumulation of interphase III nuclei — to use the terminology of PATAU and SWIFT (1953) i. e. nuclei which have synthesized the full pre-prophase amount of DNA. Since both features are reflections of mitotic activity there is no basis for assuming that TA has induced abnormal degrees of polyploidy except for one finding—the presence of high intermediate values. One was found above class IV (16n) in rat No. 53—R—92 (Fig. 7) and one above class V (32n) in rat No. 53—R—177 (Fig. 12). Additional nuclei of similar size and DNA content were found in these two rats during the special search for large nuclei (Figs. 10 and 15). Although the number of these nuclei is admittedly too small to be considered as conclusive evidence in support of ploidy induction, their position is nonetheless strongly suggestive of the occurrence of such a process. Taking for example the large intermediate in No. 53—R—92 (Fig. 7), since it is above class IV it must be concluded that it is either a highly polyploid nucleus with an aneuploid chromosome complement, or that it is a 16 ploid nucleus in interphase II (undergoing DNA synthesis).

If we assume that it is the latter, then we can conclude that class IV consists not only of class III (8n) nuclei in interphase III, but also of bona fide 16 ploid (class IV) nuclei. If this is indeed the case we have to conclude that mitosis and induction of polyploidy are taking place concurrently. That ploidy levels can be raised during active liver mitosis through merging of chromosomes of binucleate cells was demonstrated by BEAMS and KING (1942), as mentioned above. But whether such a process actually takes place in the TA livers cannot be decided with the data at hand. KLEINFELD (1952) has suggested that the 'fusion' of interphase nuclei she observed in TA rat livers might be responsible for the production of higher than normal ploidy degrees. However since such bilobed nuclei were observed in normal livers during the course of this investigation and in normal mouse livers by WILSON and LEDUC (1948, 1950a) they cannot be attributed solely to the influence of TA. Furthermore it should be pointed out that opinions differ as to the exact interpretation to be given to such nuclear configurations. Some investigators claim that they are nuclei in the process of 'fusion' (DAWSON 1940), others believe them to represent a stage in 'amitosis' (PFUHL 1938) (see WILSON and LEDUC 1948, 1950 a for discussion). Such confusion makes obvious the necessity for further combined cytological, cytochemical and karyometric investigations of the post-natal development of the normal mammalian liver before meaningful conclusions can be arrived at concerning experimental results on this organ.

With respect to BADER's (1953b) photometric DNA (Feulgen) data on mouse neoplasms from which he concluded that these tissues showed induced polyploidy, it is seen that his evidence favoring such a process is not much more substantial than that from the TA livers just discussed. Thus all the tumor DNA patterns but one display the typical characteristics of dividing tissues; viz., 1. intermediate values between ploidy modal peaks and 2. ploidy levels increased to one above the highest in the control. This, as demonstrated above, is not a reflection of induced polyploidy but stems from the accumulation of interphase III nuclei from the class below. The one pattern which suggests the possibility of induction is that of the methylcholanthrene induced squamous cell carcinoma of the rat prostate (BADER 1953b, Fig. 3) since it exhibits two classes above that of the homologous controls, in this case the normal diploid prostatic epithelium. But here, as in the TA data, the numbers are too few to carry much weight. Be it said here that for purposes of judging levels of induced ploidy the transplanted tumor such as the Cloudman S—91 melanoma and the 15091a mammary carcinoma used by BADER cannot be placed on the same comparative basis as the primary tumor with respect to tissues of origin; for it is known that following serial propagation the former can follow widely diverging evolu-

tionary paths from their tissues of origin (LEVAN and HAUSCHKA 1953). Also recently GOPAL-AYENGAR (1954) made the interesting observation that in early transplanted tumor generations of mice, rats and hamsters the cell population was consistently diploid irrespective of the ploidy relations of the tissues of origin. But later transplants have diploid, triploid and intermediate types exhibiting a gradual shift to tetraploid. In view of the inconclusive nature of the evidence on induction of polyploidy in the TA effect as well as in other pathological tissues it must be concluded in agreement with LEUCHTENBERGER, LEUCHTENBERGER and DAVIS (1954) that 1. the elevation of ploidy levels above those of homologous controls when accompanied by intermediate values is to be considered mainly as a reflection of mitotic activity; and 2. that if induction does occur it is only to an extremely limited extent.

To turn now to the interpretation of the intermediate DNA values, SWIFT (1950a and b) was the first to point out that DNA values occurring between the diploid and twice the diploid values in interphase nuclei of dividing diploid tissues were reflections of intermitotic synthesis of DNA (for review see SWIFT 1953a). Recently TAYLOR and MCMASTER (1954) have verified this by showing that the periods of DNA (Feulgen) increase per nucleus coincide very closely with the periods of incorporation of P^{32} into DNA. In accordance with the preceding findings the DNA intermediates in the TA livers have been attributed to the synthesis of DNA which accompanies the active mitosis seen in the sections, and have therefore been related to the mitotic index. It should be remembered, however, that, as pointed out by SWIFT and KLEINFELD (1953), such a correlation cannot be more than a rough approximation without a knowledge of such variables as timing of the mitotic cycle, rates of DNA synthesis and the occurrence of diurnal or other rhythms. Furthermore intermediate values may be a manifestation of an aneuploid chromosome complement resulting from abnormal mitoses such as those described in normal and treated mouse livers by WILSON and LEDUC (1950a). Also aneuploidy is known to occur to a limited extent in other normal tissues (TANAKA 1951; HSU 1952; HSU and POMERAT 1953; MANNA 1954) and to a very large extent in tumors, as shown by the chromosome counts of HAUSCHKA and LEVAN (1951, 1953), LEVAN and HAUSCHKA (1953) and the combined chemical and cytological analyses of SHELTON (1954). These findings have been corroborated by DNA (Feulgen) measurements of several investigators (CARNES, WEISSMAN and GOLDBERG 1952; PETRAKIS and FOLSTAD 1954). In addition to the above possible factors which might influence the number of intermediates there is the very real possibility that the technical difficulties involved in measuring some of the larger nuclei may have played a part.

It can be said in conclusion that, although the number of intermediate DNA values measured during TA intoxication probably exceeds the number of nuclei engaged in DNA synthesis, nevertheless their number on the average corresponds roughly to the mitotic activity observed in the sections.

B. Karyometric. Interpretations of changes in volume.

The increased variability of the TA volumes as compared to their controls causing overlapping of class extremes in the volume frequency plots (Figs. 8, 9 and 13) and the vertical spread in the volume-DNA graphs (Figs. 10, 11, 14 and 15) stems from two size variations; 1. nuclei smaller than average at the lower end of each class; and 2. nuclei larger than average at the upper end. Since, as pointed out above, volume tends to parallel protein content more closely than that of DNA, the small nuclei are probably deficient in protein and the large ones have excessive amounts of it. These differences can be interpreted in either of two ways: 1. as normal concomitants of mitosis or 2. as manifestations of a deranged protein synthesizing mechanism. In accordance with the first interpretation the graphs of the short term TA nuclei, especially that of No. 53—R—92 (Fig. 10), bear a strong resemblance to graphs similarly plotted of normal dividing tissues presented by SWIFT for root meristems of onion (SWIFT 1953b, Fig. 9) and *Tradescantia* (SWIFT 1953a, Fig. 7). In both of these tissues this author demonstrated photometrically that DNA synthesis is restricted to a limited period in interphase during which nuclear volumes show no increase. In contrast to this, protein increases are more or less continuous and are roughly paralleled by increases in nuclear volume (SWIFT 1953b, Fig. 15). In addition SWIFT showed that in division stages the protein content in these nuclei follows roughly the same pattern as that of the DNA content i. e., it is halved in amount from prophase to telophase as are also the volumes (in *Tradescantia* they are reduced to one third). It is seen from SWIFT's graphs, more clearly in *Tradescantia* than in onion, that the volume increases precede and follow DNA synthesis, hence for convenience I will name them the pre-DNA-synthesis and the post-DNA-synthesis growth periods respectively. In comparing the TA graphs with SWIFT's it is seen that the very small volumes in any one TA class, as for example in class II (4n) of No. 53—R—92 (Fig. 10), could represent newly formed post-telophase nuclei which have not yet undergone the pre-DNA-synthesis growth and the abnormally large volumes could represent diploid nuclei which have synthesized the full pre-prophase 4 C amount of DNA and have also undergone the post-DNA-synthesis growth. From this it is apparent that the situation in this class (as in every other class above the diploid) is complicated by the presence of

three types of nuclei. They are: 1. diploids which are in the terminal stages of preparation for mitosis (the larger than normal nuclei mentioned above), 2. dividing tetraploids which have either a) just completed mitosis (the abnormally small nuclei mentioned above) or b) are in the initial stages of preparation for mitosis and 3. non-dividing tetraploids. Be it said here that, although the volume range of TA nuclei showing intermediate amounts of DNA between classes I and II is of the same order of magnitude as in the corresponding classes of *Tradescantia*, the intermediates between the higher classes display a greater size range. However it must be remembered that, as mentioned above, some of these might well be reflections of aneuploidy. Thus it appears that the variability in nuclear volume in the short term rats can be interpreted in terms of mitosis as follows: 1. the larger than normal nuclei in each class are those which have completed the post-DNA-synthesis growth and 2. those that are smaller than normal are the newly formed post-telophase nuclei which have not yet undergone the pre-DNA-synthesis growth.

Although it is possible to interpret the variability in volume in the short term rats on the basis of mitosis alone, the exaggerated changes in the long term rats, especially in rat No. 53—R—177, cannot thus be accounted for. It is suggested that the extreme nuclear size variability, as shown by the marked vertical spread in the graph (Fig. 15) and the excessive overlapping of class extremes in the volume frequency plot (Fig. 13), is the reflection of a desynchronization of the DNA and protein synthesizing mechanisms. That the two can proceed independently and are influenced differently by different physical and chemical agents was demonstrated by SWIFT (1953 b). This author showed that, although both X-rays and N mustard arrest mitosis in dividing onion meristem (his Fig. 13), the former agent inhibits simultaneously the synthesis of both protein and DNA whereas the latter, the N mustard, halts only the synthesis of DNA. In these nuclei the protein content and nuclear volume continue to increase until abnormally large volumes are obtained in classes I and II — the non-DNA-synthesized (2 C) and the DNA-synthesized (4 C) respectively. Since in the TA effect under discussion similar abnormally large nuclei in each class are responsible for the extreme variability, it is suggested that TA may exert some sort of selective action on one or the other of the two synthetic processes without actually interrupting mitosis. Thus either a slowing down of DNA synthesis or a speeding up of protein production (more probably the latter) would result in the accumulation of protein in excess of the normal requirements for cell division and would lead to abnormal enlargement of the nuclei in each class, as in the case of the N mustard treated meristem.

In the preceding discussion it has been assumed that the increased volumes are attributable to increased amounts of protein. Although there are no cytophotometric Millon measurements to verify this relationship, the assumption is supported by the cytological indications that the TA parenchyma cells are engaged in strenuous protein synthesis. This can be concluded, not only from the presence of large amounts of nuclear protein (LAIRD 1953), but also from the presence of the markedly enlarged nucleolus with increased RNA content (LAIRD 1953). This combination, according to the original generalization of CASPERSSON and SCHULTZ (1939), is the inevitable concomitant of protein synthesis. CASPERSSON and his co-workers (1950) have since repeatedly emphasized this correlation and have erected an elaborate cytological system concerned with protein synthesis (for a lucid description of this see DANIELLI 1953). In his review of the more recent developments in the field of intracellular protein synthesis POLLISTER (1954) summarizes the present state of our information on the nucleolus as follows: "... although there is no evidence of the complete synthesis within the nucleus of any cytoplasmic protein, it does appear that large nucleoli and increased RNA content accompany or precede rapid protein synthesis". Recent confirmation of this view has been afforded by the demonstration (TAYLOR and MCMASTER 1954) that P^{32} incorporation into RNA (both nuclear and cytoplasmic) reaches its peak in the interval between cessation of DNA synthesis and prophase (the above described post-DNA-synthesis growth period). Proof that the nucleolus does mediate in protein synthesis has recently been afforded by the autoradiographic study of the uptake of labelleled glycine by this organelle in starfish oocytes (FICQ 1953). Thus, although the nucleus gives every indication that the TA cell is engaged in rapid protein synthesis, the cytoplasm lacks the abundant basophilic granules characteristically associated with such a process. That this is attributable to reduction in amounts of RNA is indicated by the results of chemical analysis during a 28 day period of TA administration (LAIRD 1953). However recently MIRSKY, ALLFREY and DALY (1954) have demonstrated that apparent absence of such microscopically visible granules in liver cells of starved animals and in those treated with cortisone (LOWE and WILLIAMS 1953) can simply mean that the ribonucleoprotein has become invisible through dispersion with no reduction either in its amount in the pellet material or in its ability to incorporate N^{15} labelleled glycine. On the basis of this evidence it cannot be decided whether the absence of cytoplasmic basophilia following extended TA treatment indicates loss of RNA or not. It can be said however that under such circumstances the liver cells bear a certain resemblance to those found by STENRAM in livers of protein-depleted rats (1953) and of rats deprived of plasma

albumin (1954). This author interprets the absence of microscopically visible cytoplasmic basophilia in the presence of enlarged nucleoli as evidence of a strained protein synthesizing apparatus. It could well be that the TA liver cells are under such a strain, especially if we interpret the TA effect in accordance with the recently proposed feed-back system in control of liver-mitosis (GLINOS and GEY 1954) and described in the next section. In accordance with this the protein synthesizing apparatus of the liver parenchyma cell would be burdened with the dual tasks of 1. providing cytoplasmic protein needed for cell division and 2. restoring the plasma 'protein' side of the feed-back system. It should be said here that accumulation of excessive amounts of such protein, or specific protein precursors (POLLISTER 1954), might be a contributory factor in the production of the abnormally large nuclear volumes just discussed. In summary then, since the greater nuclear size variability in the long term rats as compared to those of the shorter term stemmed from increased rather than decreased volumes, it was assumed that such increases were attributable to excessive amounts of protein arising from a) a desynchronization of protein and DNA synthesizing mechanisms superimposed upon the usual increases associated with mitosis and b) an overproduction of protein to compensate for the deficiency in the 'plasma protein' side of the feed-back system. The preceding assumption was supported by the following findings: a) increased amounts of nuclear protein demonstrated by chemical analysis and b) cytological indications that the parenchyma cell is engaged in strenuous protein synthesis.

III. Reversal. Interpretation of changes during recovery.

The questions originally set concerning the reversibility of the more severe TA effects and the relations of nuclear DNA content to the diminished nuclear volumes have been answered as follows: 1. the more severe TA effects are almost completely reversible and 2. the diminished nuclear volumes are paralleled by proportionate reductions in nuclear DNA content. During the downward shift in the frequencies of the ploidy classes the DNA distribution pattern adheres to the 1:2:4:8 of the normal liver ploidy series. Also the number of intermediates is reduced. The progressive reduction of the vertical spread in the graphs and the overlapping of class extremes in the volume frequency plots is presumably attributable as much to the reduction in mitotic activity as to the elimination of the toxic effect of the TA. The persistance in class I of a wide vertical spread in two of the graphs (Fig. 20) can be explained on the basis of the previously described fluctuation in volume normally accompanying mitosis, since the mitotic activity in these livers is confined largely to this class. That it could not possibly be

interpreted as a lingering TA effect is evinced by the findings that 1. S^{35} labelleled TA is extremely rapidly excreted (more than 80% appears in the urine during the first twenty four hours following injection) and 2. no tissues show any exceptionally great retention of radioactive sulfur after repeated injections (NYGAARD, ELDJARN and NAKKEN 1954).

The question of the reduction in the frequencies of the large polyploid nuclei and their replacement by numerous diploid and occasional haploid nuclei cannot be answered with the data at hand. The present evidence is in agreement with RATHER's (1951) conclusion that we are not dealing here with a simple regenerative process stimulated by removal of large numbers of cells through necrosis, since the concentrations of TA used in this experiment produced no visible evidence of cell damage or death. This, in conjunction with the fact that during the intoxication all ploidy classes appeared to be equally active mitotically, should have led to the restoration of normal ploidy relations during recovery rather than to a predominance of diploid nuclei. It is suggested that this reduction in ploidy levels might be through some form of chromosome reduction as originally postulated by BERGER (1938) for the changes taking place in the larval ileum of Culex. Here at the time the number of cells is greatly increased their size is correspondingly diminished as is also their ploidy valence from 64n down to 2n. Recently MERRIAM and RIS (1954) suggested that some such form of chromosome reduction might be responsible for the haploid DNA (Feulgen) values they found in the various polyploid tissues of the honeybee, even though they obtained no cytological evidence for such a process. Also suggestive of chromosome reduction is the interesting observation made recently by GOLDSTEIN (1954) that, after 24 hours in vitro cultivation of multinucleate giant cells from blood monocytes nuclei appeared in the former which were smaller than those first formed and which showed approximately haploid amounts of DNA (Feulgen). In conclusion it can be said that although the results demonstrate clearly that reversal of the severe TA effects has occurred with parallel reductions in nuclear volume and DNA content, the data are insufficient to determine the mechanism of the reversal. Only further combined cytological, karyometric and photometric analyses will answer this question.

IV. Interpretation of the TA effect and its reversal.

That recovery from the TA effect is almost complete has been clearly demonstrated. However it should be pointed out that such a reversal is not unique, since it is a well established fact that many similar chemically induced cirrhoses recover upon withdrawal of the toxic agent (SELLERS 1948).

The question of the interpretation of the TA effect and its reversal in terms of biochemistry remains for the present largely a matter of conjecture, owing to the lack of information on the precise metabolism of the compound in the body. Confining the discussion to more general levels, recent studies on the gross metabolism of S^{35} labelleled TA (NYGAARD, ELDJARN and NAKKEN 1954) show that the effect on the liver is not attributable to any specific ability of this organ to concentrate the compound. The only organs possessing this ability are the thyroid, bone marrow and adrenals but they remain unaffected. Moreover the liver is known to react in a manner similar to the TA effect following stimulation by a wide variety of chemical substances and physiological influences (SELLERS 1948; WILSON and LEDUC 1947, 1949, 1950a, b; TEIR and RAVANTI 1953; see SWIFT 1953a for others). In all such instances injury to the particularly sensitive liver parenchyma cell is manifested characteristically by mitotic activity as in the case of TA intoxication. RATHER (1951), in a discussion of the possible mechanisms by which TA might induce such cell division suggested that it might be through inactivation of growth inhibitory substances as proposed by SEXTON (1950) for its action in breaking dormancy in tubers. However since mitotic activity in the liver can be stimulated by such a wide variety of substances and conditions, a more likely explanation is afforded by the above mentioned feed-back system in control of liver mitosis (GLINOS and GEY 1954). According to this, under normal conditions, a certain concentration of unknown constituents of the blood is in equilibrium with a certain number of liver cells which produce it. A reduction in amount of this substance (STENRAM's 1954 results suggest that it is plasma albumin), either directly by plasmapheresis or indirectly through removal of liver cells, will induce and maintain liver cell mitosis until the equilibrium is restored. Since it is known that 1. TA is sufficiently toxic to inhibit growth in non-dormant tubers (SEXTON 1950) and to prevent orange decay (CHILDS and SIEGLER 1946) and 2. in higher concentrations is a severe hepatotoxin (AMBROSE, DEEDS and RATHER 1949), in low concentrations it could well inflict a degree of cell damage which, although too mild to produce visible necrosis, could nevertheless incapacitate the cells sufficiently to reduce the cell side of the above equilibrium and thus stimulate mitosis. The continued administration of minimal amounts would then not only keep mitosis going indefinitely but would fulfill the conditions necessary for the production of cirrhosis — a repeated dose of the noxious agent before the liver has had time to recover from the previous dose (CAMERON and KARUNARATNE 1936). On the above basis the TA effect can be interpreted as a continuously blocked effort on the part of the liver to compensate for the lack of functioning parenchyma cells, and the reversal as the completion of the effort upon removal of the obstructing agent.

Summary.

1. Thioacetamide administered to white rats by the drug-diet method brought about an increase in nuclear DNA content of their liver parenchyma cells in the sense that the frequencies of the higher ploidy classes were increased over those in the untreated normal liver.

2. The ploidy classes tended to show a loss of discreteness which was interpreted as being due to DNA synthesis incident to increased mitotic activity.

3. The nuclear volumes in TA treated livers maintained roughly the same proportionality to DNA content as in the controls. It is postulated that the greater variability resulted from more active mitosis combined with a deranged protein synthesis.

4. When TA treatment was stopped there was a gradual reversal of the effects just mentioned. The proportions of nuclei in the lower ploidy classes increased again and a few haploid nuclei appeared.

5. It is suggested that some type of somatic chromosome reduction is responsible for this return to normal conditions.

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Berichtigung

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Figur 1 auf Seite 284 und Figur 7 auf Seite 293 sind vertauscht; die Legenden stehen richtig.

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From the Institute of Pathology, Western Reserve University, Cleveland, Ohio.

THE POSSIBLE ROLE OF THE DNA CONTENT OF SPERMATOZOA FOR THE ACTIVATION PROCESS OF THE EGG OF THE CLAM, SPISULA SOLIDISSIMA.

By

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With 6 figures in the text.

(Eingegangen am 24. Februar 1955.)

I. Indroduction.

During natural fertilization of an egg by a spermatozoon, activation of the egg constitutes a fundamental step in the fertilization process (see for review LOEB 1913, F. R. LILLIE 1919, R. S. LILLIE 1941 and RUNNSTRÖM 1949). Since, however, activation of eggs has been introduced experimentally in the absence of spermatozoa by a variety of chemical and physical agents, the spermatozoon itself has frequently been regarded as only one of several alternative stimuli for activation (DALCQ 1928, TYLER 1941, ALLEN 1953). On the other hand, it must be kept in mind that the artificial activation in the absence of the spermatozoon in model experiments leads even in the most favorable cases only to parthenogenetic development. Consequently this cannot be compared directly with the natural activation taking place during natural fertilization (TYLER 1941) where both egg and sperm are involved.

While it is realized that a number of factors may contribute to the activation during the natural fertilization process, the possible importance of the spermatozoon itself cannot be disregarded. Some early attempts to study the possible role of the spermatozoa themselves for the activation process have been made. O. HERTWIG in 1911 demonstrated that if the chromatin of spermatozoa is damaged by irradiation, activation occurs, but species specificity is lost and no normal fertilization takes place; in other words, the damaged spermatozoon acts more or less as a parthenogenetic agent. X-irradiation experiments on *Spisula* gametes were carried out by RUGH in 1953. RUGH reported that X-irradiation of highly concentrated spermatozoa did not impair their fertilizing capacity while X-irradiation of diluted suspensions of spermatozoa lead to loss of their fertilizing ability. The work of SAMPSON (1926), JUST (1930) and FRANK (1939) has been mainly concerned with the extraction of specific activating principles from the spermatozoa, but

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these attempts were without any success. HÖRSTADIUS et al, in 1954, extracted desoxyribosenucleic acid (DNA) from spermatozoa and thymus. Testing these DNA extracts for activating properties, they found that if DNA from spermatozoa of various species of sea urchin or from calf thymus was injected into sea urchin eggs, activation was achieved but no further development took place.

The present work which is concerned with the possible role of the amount of DNA in the spermatozoa for the activation process was suggested by previous findings of LEUCHTENBERGER et al. in 1953. These workers reported that in contrast to a constant haploid amount of DNA found in the spermatozoa of fertile human males, spermatozoa of

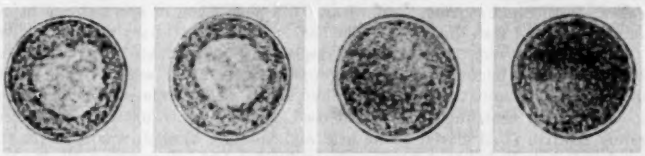

Fig. 1. Photomicrographs of living *Spisula* eggs. a, b before activation, showing intact germinal vesicle. c, d after activation, showing dissolution of germinal vesicle. × 375.

infertile human males contain significantly lower amounts of DNA. These results indicated that perhaps the amount of DNA in intact spermatozoa may be of possible importance for the fertilization process. The question arose whether the DNA content of spermatozoa may play a role during the activation process in natural fertilization. To study this problem the clam, *Spisula solidissima*, seemed an especially favorable model because the spermatozoa can be studied not only in the testis but can be readily observed after the penetration into the egg. Furthermore, the activation, as indicated by the breakdown of the germinal vesicle membrane, can easily be recognized under the microscope (Fig. 1). The amounts of DNA can be determined in individual spermatozoa in the testis as well as in the sperm nuclei within the eggs in situ under the microscope by microspectrophotometry of the Feulgen stain. The validity of this method for the estimation of DNA has been repeatedly demonstrated and discussed (see for review POLLISTER 1950, DAVIDSON 1953, SWIFT 1953, LEUCHTENBERGER 1954, 1954a).

In the following, results are presented which show that sperm nuclei within inactivated eggs contain significantly lower amounts of DNA than the sperm nuclei within activated eggs of the clam, *Spisula solidissima*.

II. Material and Methods.

The clam, *Spisula solidissima* used in this study was obtained during the summer of 1953 at the Marine Biological Laboratory, Woods Hole, Massachusetts.

The animals were kept in tanks of running sea water and the gametes were obtained by the technique described by ALLEN (1951). After evisceration, the ovaries were cut into small pieces and the eggs strained through several layers of cheesecloth into a beaker of filtered sea water. The eggs were washed by allowing them to settle to the bottom and the supernatant was siphoned off. This washing was repeated three times, over a period of one hour, and the eggs were then used for the fertilization experiments. Spermatozoa were obtained by cutting pieces of the testis into a stender dish which was then placed in the refrigerator. The spermatozoa which extruded from the testis into the dish were used within two hours for further dilution and insemination. *Spisula* spermatozoa obtained in this manner have been diluted and successfully used for fertilization experiments for periods up to six hours by ALLEN (1953) and according to RUGH (1953) may retain fertilizability for at least forty-eight hours if kept undiluted in the refrigerator at 10° C. Sufficient quantities of eggs were used to form a dense layer over the bottom of a one liter beaker. With rapid stirring, 2 ml. of an 0.5% suspension of spermatozoa in sea water were mixed with the eggs and the beaker was set on the sea table which had a temperature between 20° to 23° C. Samples of the inseminated eggs were occasionally examined microscopically at appropriate intervals between five and ninety minutes after insemination. At the desired stages of development, the eggs were pipetted into a centrifuge tube, condensed by light centrifugation, and fixed. Carnoy fixative (1 : 3 glacial acetic acid and absolute alcohol) was used for the cytochemical studies and in addition, tissues fixed in 10% neutral formalin, Zenker, or Bouin fixative and stained with iron hematoxylin or Feulgen fast green were used for the cytological studies. Paraffin sections were cut at 5 to 15 microns and stained by the standardized Feulgen method (STOWELL 1945, DISTEFANO 1948, LEUCHTENBERGER 1950). Hydrolysis in 1 N HCl for 10 minutes at 60° C was found to be optimal for this material. Estimation of the relative amounts of DNA was by the microspectrophotometric technique of POLLISTER and RIS (1947) as modified and described by LEUCHTENBERGER (1950), SWIFT (1950), and ALFERT (1950). The computation of the arbitrary amounts was done according to SWIFT (1950) and the arbitrary units were multiplied by a factor of ten.

Since all of the DNA data presented in this report were obtained by the microspectrophotometric analysis of the Feulgen stain in individual nuclei, it should be stressed again that the general validity of the method for DNA determinations in nuclei has been established by comparing it with other standard methods, such as chromosomal counts, biochemical analysis and ultraviolet microspectrophotometry (LEUCHTENBERGER 1954a).

III. Results.

The results of the DNA measurements of individual spermatozoa found in activated and inactivated eggs are presented in Fig. 2. A significant difference between the mean DNA values in the sperm nuclei of activated eggs and of inactivated eggs can be seen, namely 4.2 ± 0.06 and 2.9 ± 0.09 respectively. The value of 4.2 corresponds to the normal haploid DNA value when compared with the diploid value of 7.9 ± 0.35 in epithelial cells of the clam (Fig. 3). Furthermore, if one considers the individual distribution of the DNA values, only 13% of the sperm nuclei in the activated eggs have DNA values between 2.5 and 3.5 while the great majority of the sperm nuclei (namely 82%) within the inactivated eggs have DNA values between 1.5 and 3.5.

On the right hand side of Fig. 2, the nuclear volumes of the same sperm nuclei within activated and inactivated eggs are compared. It is

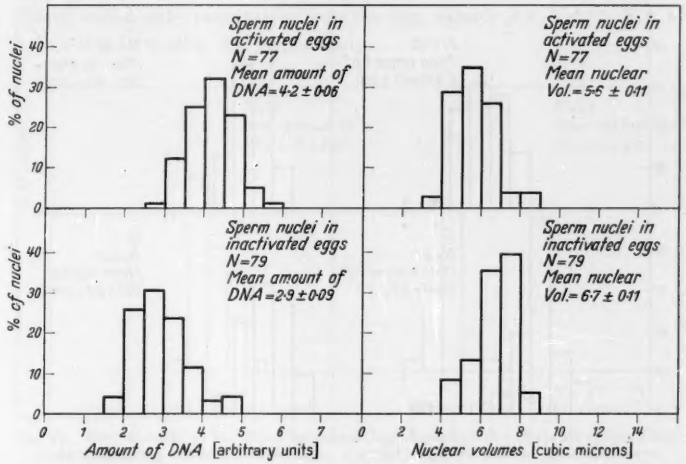

Fig. 2. Left, amount of DNA (by microspectrophotometry) in individual sperm nuclei in activated and inactivated *Spisula* eggs. Right, nuclear volumes of same specimens.

evident that the mean nuclear volume of the sperm nuclei in the inactivated eggs is slightly higher than that of the sperm nuclei in the

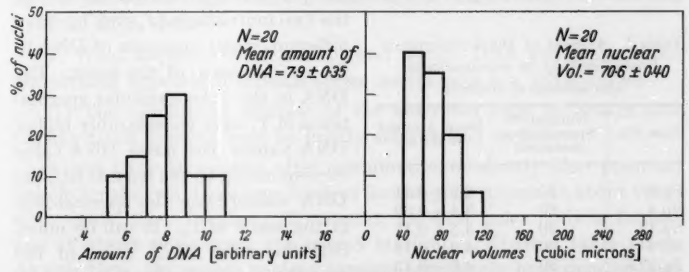

Fig. 3. Left, amount of DNA (by microspectrophotometry) in individual ciliated epithelial cell nuclei from *Spisula* testes. Right, nuclear volumes of same specimens.

activated eggs, in spite of the fact that the mean DNA value for the first group is significantly lower than that of the second group.

In order to see whether the low amounts of DNA as found in the sperm nuclei in the inactivated eggs are already present in the sperma-

tozoa before they penetrate the egg, DNA determinations were done in the spermatozoa in the testis of different individuals of the clam.

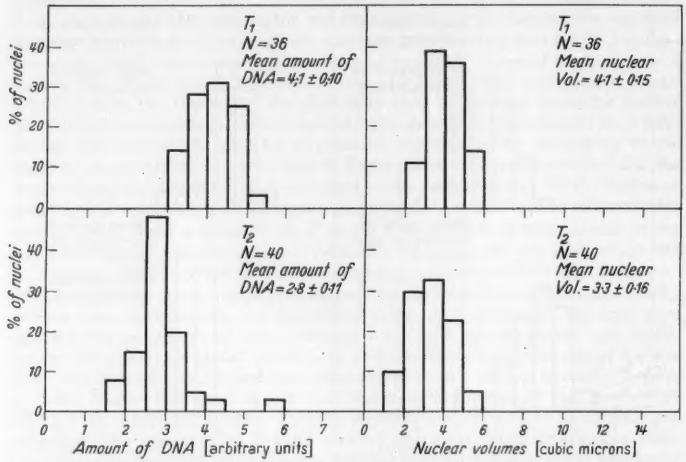

Fig. 4. Left, different amounts of DNA (by microspectrophotometry) in spermatozoa from testes of different individuals (T_1 and T_2) of the clam, *Spisula*. Right, nuclear volumes of same specimens.

In Fig. 4, the DNA values in spermatozoa from testis of two different individuals (T_1 and T_2) of the clam are presented. The data show that the two individuals (T_1 and T_2) have different mean amounts of DNA in the spermatozoa of the testis. The DNA in the intratesticular spermatozoa of T_1 have considerably higher DNA values (the mean DNA value corresponding to the normal haploid DNA value) than the spermatozoa in the testis of T_2. It can be noted that the mean DNA value of the spermatozoa of T_2 closely resembles the mean DNA value which is found in the sperm nuclei in the inactivated egg, namely 2.8 ± 0.11 (Fig. 4) and 2.9 ± 0.09 (Fig. 2) respectively. The mean amounts of DNA in intratesticular spermatozoa of five other clams were found to have either predominantly the normal haploid value or lower DNA values (see Table 1).

Table 1. *Amount of DNA (microspectrophotometry) in intratesticular spermatozoa of Spisula.*

Case No.	Number of Spermatozoa measured	Mean Amount of DNA[1]
Sp 9	40	2.8 ± 0.11
Sp 8	20	3.4 ± 0.09
Sp 12	20	3.5 ± 0.11
Sp 4	20	4.0 ± 0.12
Sp 23K	29	4.0 ± 0.12
Sp 15	36	4.1 ± 0.10
Sp 19	20	5.0 ± 0.10

[1] Expressed in arbitrary units.

In Fig. 4a the DNA values of the sperm nuclei from T_1 within activated eggs are presented. It is evident that the clam T_1 contains the same mean amount of DNA in its spermatozoa of the testis as in its sperm nuclei after penetration into the egg, namely 4.1 ± 0.10 (Fig. 4) and 4.0 ± 0.08 (Fig. 4a) respectively.

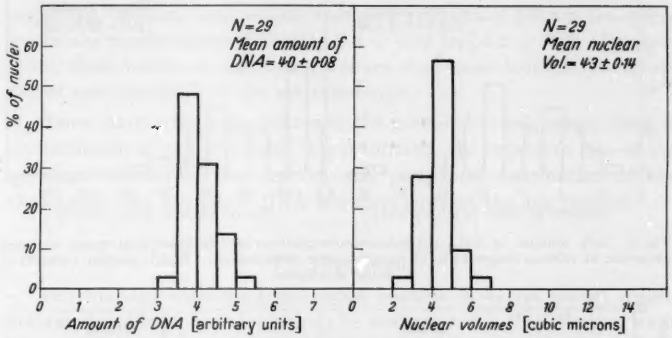

Fig. 4a. Left, amount of DNA (by microspectrophotometry) in individual sperm nuclei (from *Spisula* T_1) within activated eggs. Right, nuclear volumes of same specimens.

The next problem which seemed of interest to investigate was concerned with the following questions:

1. What happens cytologically and with reference to DNA synthesis, in the activated eggs which contain sperm nuclei with the normal haploid DNA value?

2. What happens cytologically, and to the DNA synthesis in the inactivated eggs which contain sperm nuclei with low DNA values?

(1) The cytological changes of the activated eggs in Zenker and Carnoy fixed sections may be briefly summarized as follows:

About 15 to 20 minutes after insemination or shortly after germinal vesicle breakdown, the sperm nucleus increases in volume to about twice the original size. At about 25 minutes, the first polar body is formed and at about 40 minutes, the second maturation division takes place. At this time, the sperm nucleus appears completely homogeneous and its size and shape are similar to those at 25 minutes. At about 50 minutes, that is, when the chromosomes of the egg nucleus are at late telophase of the second meiotic division, the sperm nucleus undergoes another increase in size. This increase is very rapid and in about 10 minutes, the full sized pronucleus which may be 50 to 60 times the original volume, is formed. At the same time the egg pronucleus is also

formed and at 60 minutes, the sperm and egg pronuclei are hardly distinguishable from each other.

In regard to the DNA synthesis in such activated eggs, the data presented in Fig. 5 show that at the time of pronucleus formation, the

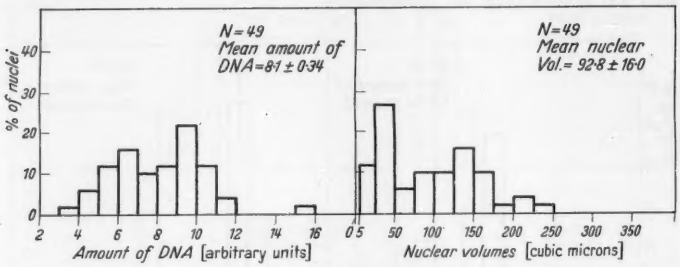

Fig. 5. Left, amount of DNA (by microspectrophotometry) in individual sperm and egg pronuclei at various stages 48 to 60 minutes after insemination. Right, nuclear volumes of same specimens.

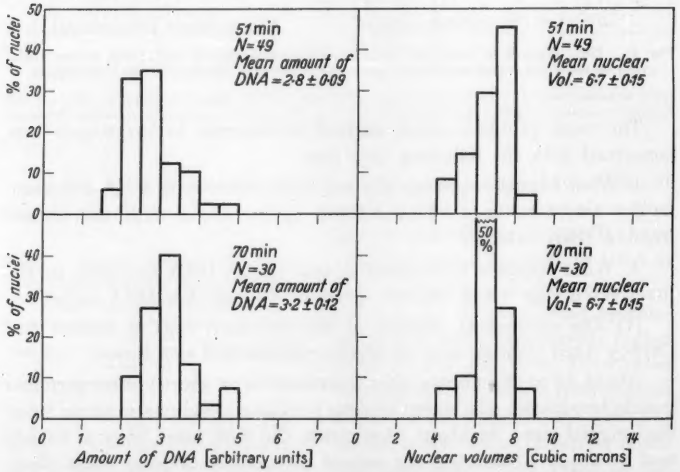

Fig. 6. Left, amount of DNA (by microspectrophotometry) in individual sperm nuclei within inactivated *Spisula* eggs at different periods after insemination. Right, nuclea volumes of same specimens.

DNA in the sperm as well as in the egg pronuclei increase from haploid value up to approximately the diploid value.

(2) The picture is considerably different if we consider the cytological changes and DNA synthesis in the inactivated eggs. The only significant

change which can be observed is the doubling of the size of the sperm nucleus which takes place within 50 minutes after insemination, in other words, neither sperm nor egg pronuclei are formed. In accordance with this cytological behavior is the lack of DNA synthesis in the sperm nuclei as can be seen in Fig. 6, where the DNA data for sperm nuclei in inactivated eggs 51 minutes and 70 minutes after insemination are presented. At both time periods, the mean amounts of DNA in the sperm nuclei are nearly identical namely 2.8 ± 0.09 and 3.2 ± 0.12. Furthermore, these values are significantly lower than those found in the sperm nuclei and pronuclei of the activated eggs.

Since inactivated eggs remain in the germinal vesicle stage, there is no formation of egg pronuclei. Unfortunately the enormous size of the germinal vesicle and the uneven and peripheral distribution of the chromatin does not allow DNA measurements of the egg nucleus.

IV. Discussion.

The findings concerning the DNA content in sperm nuclei within activated and inactivated eggs may be summarized in the following way:

1. Sperm nuclei within activated eggs contain a haploid amount of DNA which increases during pronuclei formation to approximately the diploid amount.

2. Sperm nuclei with significantly lower amounts of DNA are found within inactivated eggs and do not show any significant increase later.

3. Intratesticular spermatozoa do not contain the same amounts of DNA for different individuals of the clam, *Spisula*. Some clams show the predominantly typical haploid amount of DNA in the spermatozoa and some show significantly lower amounts of DNA.

If the findings as presented above are considered in the light of the process of nuclear activation of eggs, the occurrence of eggs which are not activated and which contain sperm nuclei with low amounts of DNA might be interpreted as a disturbance in fertilization caused by the deficiency of DNA in the sperm nuclei. The possibility that infertility may be due to low DNA in spermatozoa was previously suggested by LEUCHTENBERGER et al. (1953). These workers showed that human spermatozoa in fertile males show a strikingly uniform haploid amount of DNA while infertile males have significantly lower DNA values in their spermatozoa.

That deficiency in a component of spermatozoa may be a cause for failure to fertilize is in accordance with the observations that chromosomal and consequently a genetic imbalance may prevent normal fertilization. Since DNA has been established as a consistent component

of chromosomes (see summary in SCHRADER 1953) it seems reasonable to regard differences in amounts of DNA as reflecting differences in chromosomal material. However, chromosomal imbalance or damage has been generally considered as not affecting initial activation of the egg but a later stage leading to aborted cleavages, developmental arrest or poor viability (LOEB 1913, TYLER 1941). On the other hand, the evidence presented in this report suggests that the DNA of the spermatozoa appears to be correlated with a rather early process in fertilization, namely the nuclear activation.

Activated eggs contain spermatozoa with a normal haploid amount of DNA (Fig. 2) while inactivated eggs contain spermatozoa with a significantly lower amount of DNA (Figs. 2 and 6). Since the low amount of DNA in inactivated eggs is probably not due to a loss of DNA after insemination but can be traced back to a deficiency in the spermatozoa contained in the testis (Fig. 4) it is entirely probable that a normal haploid amount of DNA in the spermatozoa is a prerequisite for nuclear activation of the eggs, at least in *Spisula*.

The deficient amount of DNA found in spermatozoa in the testis of some individuals of the clam, *Spisula solidissima* (Fig. 4) is in good accordance with the deficient amount of DNA in spermatogenic nuclei and spermatozoa in the testis of infertile human males as reported by LEUCHTENBERGER et al. (1954). Since already the primary and secondary spermatocytes and spermatids of infertile human males have significantly lower amounts of DNA than primary and secondary spermatocytes and spermatids of fertile human males, the DNA deficiency found in the spermatozoa can be traced back to a much earlier stage in DNA synthesis. Preliminary studies indicate that when spermatozoa with low amounts of DNA are present, deficient amounts of DNA are already present in the primary and secondary spermatocytes in the testis of the clam. An extensive investigation of this problem is under way.

That a normal DNA content in spermatozoa may not only be essential for activation, but may even be of significance for the penetration of the spermatozoon into the *Spisula* egg is suggested by the following observation:

If a group of eggs is exposed to spermatozoa with predominantly normal haploid DNA content, approximately 98% (1513 out of 1546) of the eggs are activated and all the activated eggs measured contained a normal haploid amount of DNA. If another group of eggs is exposed to a suspension of spermatozoa with a predominantly low DNA content (only 10% of the spermatozoa measured had the normal haploid DNA value) the percentage of activated eggs is surprisingly high, namely

78% (669 out of 856). All of the sperm nuclei within this group of activated eggs measured had the normal haploid DNA content. This finding suggests that the small percentage of eggs containing spermatozoa with the haploid amount of DNA succeeds in penetrating a large number of eggs while the spermatozoa which contained the abnormal amount of DNA penetrated only a relatively small number of eggs and thus the shift in percentage is explained. It seems that the spermatozoa with a normal haploid amount of DNA have a selective advantage for penetrating eggs over those spermatozoa which have a low amount of DNA. However, the DNA deficiency in a spermatozoon does not completely prevent its penetration into the egg as indicated by the inactivated eggs with sperm nuclei containing low amounts of DNA, but probably may only hinder its ability to enter.

Although this study has been concerned predominantly with the role of the spermatozoa for the activation process, it is realized that the egg itself may have at least the same importance for the activation process. As a matter of fact, inactivated eggs have usually been interpreted as being due to some disturbance of the egg itself, such as, "underripeness" or "overripeness" of the egg (RUNNSTRÖM 1948, 1949).

Furthermore, it is reasonable to assume that some eggs may also have faulty amounts of DNA as found in some spermatozoa. If we accept the concept that a deficient amount of DNA does contribute to a disturbance of the activation process, then an egg with a low amount of DNA may also lead to inactivation of the egg, even if it is penetrated by a spermatozoon with the normal haploid amount of DNA. Unfortunately the DNA content in the eggs of the clam, *Spisula* before pronuclei formation cannot be readily measured due to the uneven distribution of the chromatin as discussed earlier. On the other hand, some of the DNA data presented in this report give support to the possibility that there may be eggs with faulty amounts of DNA. If we examine the DNA data in Fig. 2 for the sperm nuclei in inactivated eggs, it can be noted that a certain number (about 18%) of inactivated eggs contain sperm nuclei with normal haploid amounts of DNA. One can hardly escape the conclusion that among this group, some of the eggs were faulty in some way, either possibly being "underripe" or "overripe" or perhaps containing abnormal amounts of DNA.

On the basis of the data presented, the quantitative aspects of DNA in spermatozoa for the activation process of *Spisula* eggs have been discussed. The findings that normal haploid amounts of DNA were present in the sperm nuclei of all activated eggs while lower amounts of DNA were present in sperm nuclei of inactivated eggs seem to suggest that the constant haploid amount of DNA in spermatozoa may be of significance for the activation process.

Summary.

1. Sperm nuclei within activated eggs of the clam, *Spisula solidissima* were found to contain a normal haploid amount of DNA while sperm nuclei in inactivated eggs of the same clam contain significantly lower amounts of DNA.

2. The DNA content of sperm nuclei within activated eggs increases to approximately the diploid amount during pronuclei formation whereas the DNA content of sperm nuclei in inactivated eggs does not show any increase.

3. The amount of DNA in spermatozoa in the testis of *Spisula* varies from one clam to another. Some clams have predominantly the normal haploid amount of DNA while others show significantly lower amounts of DNA in the spermatozoa in the testis.

4. The possible significance of the DNA content of spermatozoa for the activation process of the *Spisula* egg is discussed.

We wish to thank Professor FRANZ SCHRADER and Dr. SALLY HUGHES-SCHRADER of Columbia University, New York for their many helpful suggestions and discussions particularly in connection with the cytological aspects of this problem.

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CHROMOSOMAL CONTROL OF NUCLEAR COMPOSITION IN MAIZE*.

By

MEI LIN.

With 13 figures in the text.

(Eingegangen am 28. Februar 1955.)

I. Introduction.

The nucleolus is a cell organelle which is almost universally present in the nuclei of living organisms, with the exception of a few highly differentiated types such as spermatozoa (see however SOTELO and TRUJILLO 1954), certain leucocytes of mammals, and certain echinoderm eggs and pregastrula embryos (CASPERSSON 1950). Because of its wide occurrence, it has long been the object of attention among cytologists, and there have been a number of reviews dealing with the literature on the nucleolus, notably by MONTGOMERY (1898), LUDFORD (1922), DERMEN (1933), GATES (1942a), and VOGT and VOGT (1947).

Historically, the nucleolus was first observed in the epithelial cells of the eel by FONTANA in 1781 (quoted by HENNEGUY 1896). The term "nucleolus" was first used by BOWMAN (1840), while the term "plasmosome" was introduced by OGATA (1883) to distinguish the true nucleolus (FLEMMING 1882), which is Feulgen-negative, from the karyosome (OGATA 1883), or chromatin nucleolus of MONTGOMERY (1898), which is actually nothing more than a thickening of chromatin and is consequently Feulgen-positive. Terminology in the early literature is somewhat confusing because of the indiscriminate use of the term "nucleolus", but in the current literature this term has been confined to the plasmosome or true nucleolus.

Physical nature.

For all its diversified occurrence, the nucleolus is surprisingly constant in its appearance. It is perhaps the most conspicuous structure in the interphase nucleus. The number of nucleoli arising in telophase can now be regarded as characteristic of the species (see below). However, if more than one arise in telophase they often fuse before the following prophase into a single highly refractile body which is ordinarily spherical in shape. MONTGOMERY (1898) discusses the comparative cytology of nucleolar number.

The nucleolus is generally thought to be liquid, or at least semi-liquid (WILSON 1925), and to be without any limiting membrane (BORYSKO and BANG 1951; YASUZUMI and YAMANAKA 1952). However, DURYEE (1950) in his work on amphibian eggs spoke of a nucleolar membrane, while CHAYEN (1952) observed the

* This paper, which is LIN's thesis for the Ph. D. degree, is being published posthumously. Dr. LIN's sudden and tragic death occurred in September of 1954. M. M. RHOADES.

presence of a welldefined "skin" surrounding the nucleolus in the root tips of *Vicia faba*. It has been suggested that the physical nature of the nucleolus is that of a coacervate (HYGÉN 1941, EHRENBERG 1946, DURYEE 1950). Under certain conditions it is possible for two aqueous solutions of the same colloid, one dilute and the other concentrated, to be immiscible with each other, a phenomenon known as coacervation. It is greatly influenced by inorganic ions. HYGÉN found that the nucleoli of the egg nucleus in *Micrasterias* fused under the influence of KNO_3 while DURYEE reported that a number of reagents would dissolve the nucleoli of the oocyte of the frog. This concept, of course, depends on the question whether the nucleolus is in fact in a liquid state. As pointed out by HUGHES (1952), the physical state of the nucleolus probably varies in different organisms. In vertebrate cells in tissue culture, the nucleoli appear to be solid. VINCENT (1952), working on nucleoli isolated from starfish oocytes, concluded that the nucleolus was a solid body (at least after isolation), which did not flatten under pressure but fragmented into pieces.

Among the nuclear structures, the nucleolus appears to have the highest specific gravity. GRAY (1927) found that nucleoli of the large nuclei of *Asteria* or *Echinus* oocytes moved freely through the nucleus to rest on the "floor" of the nucleus due to gravitational force. BEAMS (1943) was able to stratify by ultracentrifugation nuclei of many kinds of cells including grasshopper Malpighian tubes, *Paramecium* macronuclei, and the mesenchyme tissue of the developing chick, into several layers with the nucleoli at the centrifugal pole. More recently, VINCENT (1952) was able to separate the nucleoli of starfish oocytes from the rest of the cell by straining the eggs through cheesecloth, followed by homogenization of the filtrate and repeated centrifugation at 375 g. The density of the isolated nucleoli measured in sucrose solution was found to be 1.35–1.4. Similarly, nucleoli were isolated from rat liver nuclei by differential centrifugation (LITT, MONTY and DOUNCE 1952). GATES (1942b), however, failed to achieve separation of the nucleoli in *Mactra* eggs at a centrifugal acceleration of 70000 g.

Vacuoles are very commonly found inside the nucleoli, but their significance in the structure and function of the nucleolus is still obscure. The situation is best summarized by the following statement from DERMEN (1933): "Vacuolation was one of the most important features observed in living as well as in fixed material. At times the nucleoli in all the stigma hairs were found to be vacuolated; at other times only part of them were vacuolated; and at still other times there were stigmas with hairs none of which contained vacuolated nucleoli. It is apparent that vacuoles are not permanent features of the nucleoli but vacuolation may be considered a normal phenomenon and vacuoles may appear and disappear normally." The vacuoles, however, are described as discrete, permanent structures by certain authors. SERRA and QUEIROZ LOPES (1945) found spherical "inclusions" in the nucleoli of oocytes of certain snails. These inclusions were not displaced by centrifugation at 3000 rpm. and were only slightly affected by treatment with saline which was five times as concentrated as normal saline. This result was interpreted as an indication that they were not aqueous vacuoles. Granules in nucleoli were classically called nucleolini, see MONTGOMERY (1898). DURYEE (1950) found that dissolution of the nucleoli of certain amphibian oocytes took place in stages, the outer shell disintegrating first, freeing one or more inner droplets. He also reported that the contained vacuoles or granules were released upon the breakdown of the nucleolar membrane. CHAYEN (1952) found that the nucleoli in the broad bean consisted of an outer zone and an inner core, and that when two nucleoli were fused, two cores were found in the fusion nucleolus. RNA was found to be confined largely to the outer zone. ESTABLE and SOTELO (1952), by

means of phase contrast microscopy, dark field and oblique illumination, electron microscopy, and specialized staining procedures involving silver impregnation and iron-pyrogallic stain, were able to demonstrate the presence of filamentous structures, the nucleolonemata, within the nucleoli of cat and human neurones, oocytes of crustacean, and spermatocytes of *Laplatacris dispar*. The nucleolonema does not disappear during cell division; SOTELO and TRUJILLO (1954) suggest "Omnis nucleolonema e nucleolonema".

Size.

Under comparable conditions, the nucleoli of any one type of cell appear to have approximately the same volume. The immediate factors that affect the size of the nucleolus are the type of tissue in which it is found and the physiological condition of the cell. In general, embryonic and meristematic cells have conspicuously large nucleoli. VAN CAMP (1924) found the nuclear volume: nucleolar volume ratio in *Clivia miniata* to be 16:1 in the meristem, 30:1 in root cap cells, and 40:1 in fully mature cells. The length of the resting period since the last mitosis has a marked effect on nucleolar size. When nuclear division is delayed, the nucleoli become much larger than normal. This was found to be true for *Crepidula* (CONKLIN 1912) and *Peperomia blanda* (FISCHER 1934). The latter author further found the nucleolar size to be greatly influenced by nutrition. The nucleoli become smaller in leaves kept in darkness and show normally a diurnal size variation of 26–50%, with the highest in the afternoon and the lowest in the early morning, attributed possibly to variation in the sugar content. The author has not seen direct evidence that plant nucleoli have such a high carbohydrate content. MEYER (1918) measured the nucleolar volume in *Galtonia* in July, November and December; he found a ratio of 15:30:34 and concluded that the nucleoli contained reserve materials. Among animal tissues, the basophilic, protein-forming cells usually have large nucleoli, an observation in support of the theory of protein synthesis of the CASPERSSON school (CASPERSSON 1950). In amphibian eggs there are large numbers of nucleoli, possibly related to yolk formation (reviewed by CALLAN 1952), but in the embryos, nucleoli do not reappear until gastrulation when RNA and protein syntheses increase (BRACHET 1952). Regenerating mosses have smaller chloroplasts but larger nuclei and nucleoli and smaller nuclear/nucleolar ratios than in non-regenerating cells (HEITZ 1925). Tumor cells contain larger nuclei and nucleoli than normal cells, although the wide variation makes diagnosis by this means difficult; the nuclear/nucleolar volume ratios for normal and malignant cells do not differ significantly (GUTTMAN and HALPERN 1935). It appears that the size of the metamitotic nucleolus is affected by nutritional aspects of the cell, being larger for cells with higher metabolic activities. For such active cells the nuclear/nucleolar volume ratio may be lower than for less active cells. With metabolic activity, the volume of the nucleolus has a greater variation than has the volume of the nucleus.

Relationship to the chromosomes and the mitotic cycle.

It is now well established that the nucleoli arise in telophase at specific loci on chromosomes usually having a satellite or secondary constriction, and that they disappear usually in late prophase. The elucidation of the relationship between nucleoli and chromosomes is due largely to the classical work of HEITZ (1931) and MCCLINTOCK (1934), although PFITZNER (1882) and FLEMMING (1882) earlier noted the growth of the nucleolus as the chromosomes become less apparent and *vice versa*, and suggested a functional relationship.

Working on different species of *Crepis*, HEITZ arrived at the following conclusions: (1) The nucleoli arise at specific loci of specific chromosomes, and only at such localities; (2) the number of nucleoli arising in telophase is always constant and is as characteristic of the species as is the chromosome number; (3) when two SAT-chromosomes are present in a monoploid complement, distinctive size differences of nucleoli could result, and this would be correlated with different functional activities of the SAT-chromosomes; and (4) fusion of such nucleoli could occur very early in telophase.

HEITZ thought that the nucleolus was formed by the stalk of the satellite, and it remained for MCCLINTOCK to point out that for *Zea* the nucleolus originated not from the stalk of the satellite but from a deep-staining organized body, which she called the nucleolar-organizing body or element, directly adjacent to the stalk. *Zea* typically has two nucleoli in somatic telophase cells. MCCLINTOCK obtained a strain of maize with a reciprocal translocation involving chromosome 6, on which the nucleolar-organizing body (hereafter referred to as the nucleolar organizer) is situated, and chromosome 9. The break in chromosome 6 occured through the nucleolar organizer, so that the two interchanged chromosomes, 6^{9} and 9^{6}, each possessed a section of the nucleolar organizer capable of organizing a nucleolus. Plants homozygous for the interchange developed four nucleoli in their somatic telophases, while plants heterozygous for the interchange developed three. She further found that the functional capacity to develop a nucleolus was not the same for both segments of the severed nucleolar organizer. The segment possessed by chromosome 9^{6}, although smaller in size than the segment possessed by chromosome 6^{9}, produced the larger nucleolus. The observation that the chromosomes, especially in telophase I and prophase II, were surrounded by a layer of faintly-stained matrix material, and that the nucleolus in prophase II was confluent with the matrix material of many or all of the chromosomes, led MCCLINTOCK to postulate that the function of the nucleolar organizer was to organize the nucleolar material present in all the chromosomes into a definite body, the nucleolus. In the absence of any nucleolar-organizing element, the nucleolar substance would simply accumulate into droplets. It might be pointed out, in this connection, that similar observations have since been made in several different genera of plants such as *Narcissus* (SIKKA 1940), *Crocus* (PARTHAK 1940), and *Cassia* (JACOB 1940). In these plants there is an evanescent stage of the somatic telophase in which numerous small angular or globular bodies are scattered throughout the daughter nuclei. They stain green, like nucleoli, in Feulgen-light green, and appear to be formed from the sheath or matrix of chromosomes which also frequently appears as a green line around the red chromosomes in metaphase. In early telophase this sheath breaks up and disappears as the true nucleoli grow from their various loci.

Other evidence for a relationship between the nucleolus and the chromosomes comes from a study of polyploids. DE MOL (1926) showed an increase of "simple" nucleolar number or total nucleolar volume with degree of ploidy. From a study of aneuploids, he suggests that nucleoli are associated with particular chromosomes rather than with the total chromosome mass. BHATIA (1938) found that the total nucleolar volume in hexaploid wheat was approximately three-halves of that in the tetraploid series. Similarly, PARTHASARATHY (1938) found a ratio of slightly over 2:1 between the mean nucleolar volumes in root tip nuclei of diploid and haploid rice, and PARTHAK (1940) obtained an approximately 2:1 ratio for the nucleolar volumes of tetraploid and diploid sectors of the same root in *Crocus sativus*. However, RAMANUJAM (1937) found no significant difference in the nucleolar volumes of triploid and diploid rice, and DERMEN (1933) failed to detect a simple correlation between the size of the nucleolus and the number of chromosomes in *Petunia*

polyploids. Different diploid strains of *Petunia* had different nucleolar volumes; the degree of genetic correspondence of the polyploid series might affect the simplicity of the ratios of nucleolar volumes to the degree of ploidy. The use of nucleoli in the analysis of the origins of polyploidy is excellently reviewed by GATES (1942). KOSTOFF (1949) showed that the ratios in diploid versus tetraploid *Taraxacum* cells for nuclei was 1/2.77, for nucleoli was 1/2.44, and for the nuclear/nucleolar ratios was 7.24/8.22. BEATTY (1949) found that the sum of the areas of the nucleoli in a nucleus was proportional to the degree of ploidy in *Rana temporaria* tadpoles. It appears that the size of the nucleolus is affected not only by metabolic activity but also by the degree of ploidy of the cell, which in turn affects metabolism. The nuclear/nucleolar volume ratio apparently becomes larger with increasing ploidy. The volume of the nucleus has a greater variation with degree of ploidy than has the volume of the nucleolus.

Chemical composition.

Until 1940, what vague and conflicting knowledge was available regarding the chemical nature of the nucleolus was gained exclusively through staining reactions (for a review of the earlier work, see MONTGOMERY 1898 and GATES 1942a). The nucleolus is generally basophilic, but may under certain conditions be acidophilic. Furthermore, the degree of basophilia or acidophilia varies both with the fixative and with the particular dye combinations and conditions (GERSCH 1940). The application of the Feulgen reaction to the nucleolus has established the fact that the nucleolus does not contain a detectible amount of desoxyribonuclei acid.

A great advance in our understanding of the chemical composition of the nucleolus came with the pioneer work of CASPERSSON on the development of the ultraviolet microspectrophotometric technique, i.e., the application of ultraviolet spectrophotometry to cytological material through a microscope. (For a detailed account of this method, see CASPERSSON 1947, 1950.)

Two groups of compounds commonly found in cellular structures have absorption peaks in the middle ultraviolet wavelengths: the nucleic acids, with a maximum around 2600 Å due to the conjugated unsaturation of the purine and pyrimidine rings, and the proteins, with a maximum around 2800 Å due to the three cyclic amino acids tryptophan, tyrosine and phenylalanine. By means of microspectrophotometry in conjunction with the Feulgen reaction, CASPERSSON and SCHULTZ (1940) were able to demonstrate the presence of ribonucleic acids and proteins in the nucleoli of sea urchin eggs, *Drosophila* salivary gland cells, and spinach root tip cells. Treatment with ribonuclease has been shown to decrease both the ultraviolet absorption of the nucleolus and its affinity for basic dyes (BRACHET 1942).

The nature of the protein component of the nucleolus has been a matter of considerable dispute. The CASPERSSON school maintains that the proteins in the nucleolus are of the basic type (histones). This was confirmed by SERRA and QUEIROZ LOPES (1945) who, on the basis of results obtained by the arginine reaction, concluded that the proteins in the nucleolus of the oocyte of the snail were principally of the basic type. However, the evidence on which the tenet of the CASPERSSON school is based, i.e., a displacement of the protein absorption peak from 2800 Å to longer wavelengths, was questioned by MIRSKY and POLLISTER (1943), who pointed out that the peak for relatively pure histone preparations was in the usual protein region and not shifted toward the longer wavelenghts. CASPERSSON (1947) explained this apparent discrepancy by asserting that the shift occurred only in the presence of nucleic acids. POLLISTER and RIS (1947), in microspectrophotometric measurements of the total protein and non-histone protein by a modified Millon

reaction, found that the nucleolus of maize microsporocytes at pachynema contained no appreciable amount of histone. Likewise, VINCENT (1952) found no trace of histone in the nucleoli isolated from starfish oocytes.

Only two cases of direct chemical analysis of nucleoli have been reported. VINCENT (1952) found the main structural component of nucleoli isolated from starfish oocytes to be a phosphoprotein, the nitrogen of which made up 16–20% of the dry weight, while ribonucleic acids made up 2.2–4.6% of the dry weight. Some lipids and soluble nucleotides and the enzyme acid glycerophosphatase were also found. LITT, MONTY and DOUNCE (1952) found 17–18% desoxyribonucleic acids and only a low percentage of ribonucleic acids in nucleoli isolated from rat liver nuclei. Most of the nucleoli were described as broken from chromosomes to which they were presumably attached, but a small percentage were still attached to the chromosomes. Since nucleoli in cytological preparations of rat liver are universally Feulgen negative, the presence of desoxyribonucleic acids is very likely due to contamination, as pointed out by SWIFT (1953).

Despite the large number of investigations dealing with the chemical nature of the nucleolus, quantitative results are given in only a few. Besides the work of VINCENT cited above, POLLISTER and RIS (1947) estimated the total protein in the whole nucleolus of maize microsporocytes to be 5.0×10^{-11} grams. In another studa on the nucleotide content of maize nucleoli, POLLISTER and LEUCHTENBERGER (1949) made measurements of the absorption at 2537 Å, using as blanks sections treated with ribonuclease and cold and hot trichloracetic acid. Approximately one half of the extinction was thus found to be due to ribose nucleotides. Since about one fourth of this extinction can be reduced by cold trichloracetic acid treatment, they suggested that this part of the extinction was due to a lower nucleotide polymer than the nucleic acids.

Besides ribonucleic acids and proteins, a third type of compound, the lipids, has occasionally been reported to be present in the nucleolus. SHINKE and SHIGENAGA (1933) and MENSINKAI (1939) found lipids in certain marine eggs, while, according to SERRA (1947), in the nucleoli of snail oocytes there are "peripheral inclusions which give a coloration of middle intensity with BZL blue". GATES (1942b), however, denied that lipids were present in the nucleolus of *Asterias* and *Mactra*.

Since the intimate relationship between the nucleolus and the chromosomes has been established, the question naturally arises as to whether the chemical composition of the nucleolus is under direct genetic and chromosomal control. In a series of interesting experiments, SCHULTZ, CASPERSSON and AQUILONIUS (1940) obtained ultraviolet absorption spectra for salivary gland nucleoli from different stocks of *Drosophila melanogaster* carrying translocations involving the X and Y chromosomes. It seems clear from their results that the curve shape and the ratio of the extinctions at 2600 Å and 2800 Å are different in the different stocks, indicating a stock-specific difference in the composition; but beyond this, their results do not seem to justify any more specific, clear-cut conclusions. However, the approach does seem to be interesting and useful, as is amply shown in the present investigation.

The purpose of the present investigation is to determine the general composition of the nucleolus in maize, and to see how it is affected by various changes in the chromosomal constitution of the plant. It is hoped that the results obtained will help us gain a better insight into the nature and function of the nucleolus.

II. Materials.

Zea mays is one of the few organisms whose chromosome morphology is very well studied and in which a large number of chromosome rearrangements are available, and it is consequently an excellent material for the investigation of the relationship between the nucleolus and the chromosomes.

Among the different tissues of the plant, the microsporocytes are especially favorable for this study since in this tissue one can be certain that all the cells have the diploid number of chromosomes, whereas in other mature tissues there is always the possibility of the presence of polyploid cells (SWIFT 1950a). Save for rare exceptions, the root tip also contains only diploid cells, but here the chromosomes are being duplicated during the interphase; furthermore, in the interphase nuclei as well as in the prophase nuclei, the two nucleolar chromosomes of the diploid complement quite often produce two separate nucleoli. In the microsporocytes, on the other hand, because of the pairing of homologous chromosomes, there is almost invariably only one single nucleolus in each nucleus at pachynema or later stages of microsporogenesis. Nucleoli of early prophase microsporocytes of maize have been studied microspectrophotometrically by POLLISTER and RIS (1947) and POLLISTER and LEUCHTENBERGER (1949).

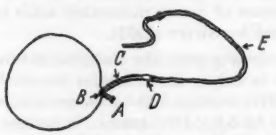

Fig. 1. Camera lucida drawing of normal chromosome 6 at pachynema. *A* Satellite. *B* Nucleolar organizer. *C* Chromatic thread joining the nucleolar organizer and the centromere. *D* Centromere. *E* Long arm with one knob. ×900.

Of the ten chromosomes in the haploid complement, only chromosome 6 is provided with a nucleolar organizer. This chromosome at pachynema (Fig. 1) can be seen to be made up of the following parts (MCCLINTOCK 1934): (1) the satellite proper, (2) a thin, practically colorless thread joining the satellite to (3) the nucleolar organizer, a large, deep-staining body conjoined to the nucleolus, (4) a chromatic thread joining the nucleolar organizer to the centromers, and (5) the long arm of the chromosome, usually with one or two knobs.

In maize, supernumerary chromosomes known as B-type chromosomes, which appear to be genetically inert (RANDOLPH 1928, 1941), are known to undergo non-disjunction at a high rate in the division of the generative nucleus (ROMAN 1947), so that by repeated selfings, the number of B chromosomes can be readily built up. The B chromosomes provide a means whereby the number of nucleolar organizers in the nucleus can be increased. A stock carrying a heterozygous reciprocal translocation between chromosome 6 and a B chromosome,

obtained from ROMAN, was used in the present investigation. The point of breakage in chromosome 6 was within the nucleolar organizer, while that in the B chromosome was in the main heterochromatic segment, approximately one third of the distance from its distal end (Fig. 2). Both portions of the severed nucleolar organizer were seen to be attached to the nucleolus, hence, the functional activity of the nucleolar organizer does not, as MCCLINTOCK (1934) had observed, depend on its intactness. Among the selfed progeny of such a plant were plants carrying from one to three B^6 chromosomes and two normal chromosomes 6. When one of the plants carrying three B^6 chromosomes was again selfed, there were found among the F_2 progeny plants carrying from none to six B^6 chromosomes. All B^6 chromosomes were found to be attached to the nucleolus at pachytene.

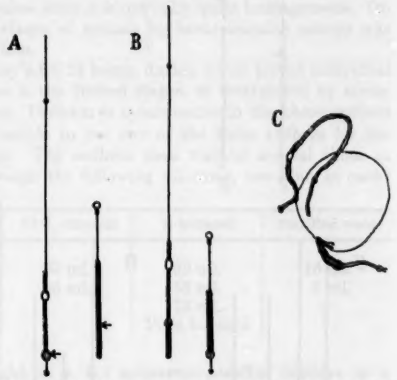

Fig. 2A–C. Chromosome constitution of the translocation involving chromosome 6 and a B chromosome. A Diagram of a normal chromosome 6 and a B chromosome. B Diagram of the interchanged chromosomes 6^B and B^6 (After ROMAN). C Camera lucida drawing of two chromosomes 6 and two chromosomes B^6, both associated with the nucleolus, at pachynema. ×900.

Before any effect observed in the chemical composition of the nucleolus in plants with extra organizers could be ascribed to these organelles rather than to the redundant segments of B chromatin present in B^6 chromosomes, it was necessary to make a comparison of these results with those obtained in plants with intact supernumerary B chromosomes and possessing only two nucleolar organizers. Fortunately, isogenic strains differing only in the presence or absence of B chromosomes were available. One plant with eight B chromosomes and one with none were obtained, thus permitting a test of the effect of B chromatin on nucleolar constitution.

Three other stocks of different chromosomal constitution were also used: one was trisomic for chromosome 6, the other a triploid and the third carried a heterozygous translocation involving chromosome 6 and chromosome 2. The break in chromosome 6 occurred in the long arm, about one tenth of the distance from the end, while the break in chromosome 2 occurred in the long arm, about one twentieth of the distance from the centromere. Plants carrying this translocation in homozygous

and heterozygous conditions, as well as plants carrying only the two normal chromosomes 6 and chromosomes 2, were obtained by selfing.

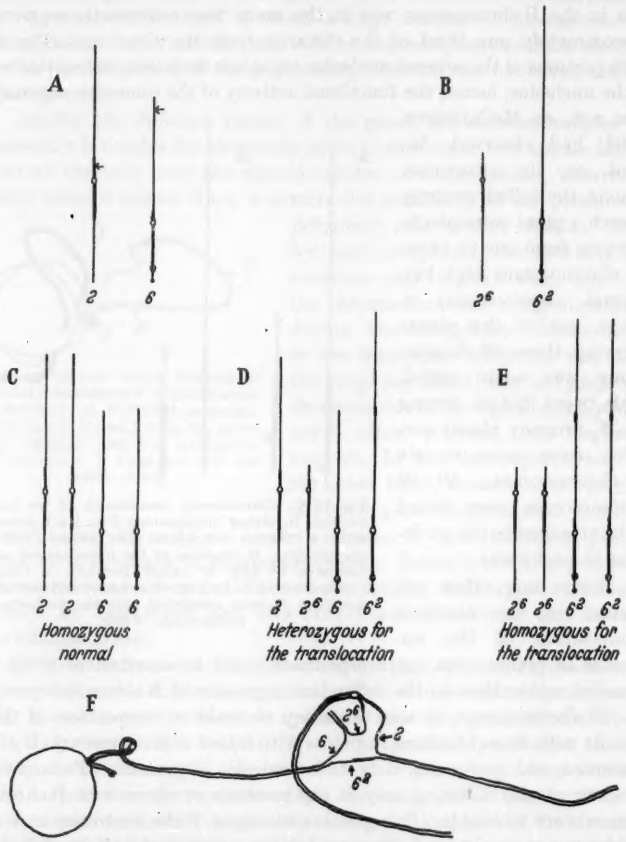

Fig. 3A–F. Chromosome constitution of plants obtained by selfing of a plant carrying a heterozygous 2–6 translocation. A Diagram of the normal chromosomes 2 and 6 (after RHOADES). B Diagram of interchanged chromosomes 2^6 and 6^2. C, D and E Chromosome constitution of the three types of plants obtained from the selfing. F Camera lucida drawing of a heterozygous 2–6 translocation at pachynema. ×900.

The chromosome constitution of these plants is shown in Fig. 3. It can readily be seen that the plant carrying the translocated chromosomes 6^2 in the homozygous condition had a much longer segment of chromosome associated with the nucleolar organizer than is normally the case.

III. Preparation of slides.

All materials used in these investigations were fixed in Carnoy's fluid (three parts absolute ethanol to one part glacial acetic acid). In microspectrophotometry, two fixatives have been most frequently used, namely, Carnoy's fluid and formalin. The former has been known to cause clumping of nucleoprotein masses in the nucleus, thus increasing the distributional error inherent to a certain extent in all microspectrophotometric studies (SWIFT 1953). However, this drawback is not serious in the case of the nucleolus since it is optically quite homogeneous. On the other hand, identification of stages of meiosis by aceto-carmine smears was greatly facilitated by Carnoy fixation.

The tassels were fixed in Carnoy's for 24 hours, during which period individual anthers containing microsporocytes in the desired stages, as determined by aceto-carmine smears, were dissected out. Division is synchronous in the three anthers of each floret; it was therefore feasible to use one of the three anthers for the identification of the division stage. The anthers were washed several times in 80% ethanol and then passed through the following solutions, one hour in each:

	Absolute ethanol	95 % ethanol	n-butanol	Distilled water
Solution 1	—	80 ml.	35 ml.	15 ml.
Solution 2	—	45 ml.	55 ml.	5 ml.
Solution 3	25 ml.	—	75 ml.	—
Solution 4	—	—	Pure butanol	—

The anthers were left overnight in a 1:1 n-butanol-paraffin mixture in a 56° C. incubator, and the next morning changed to pure paraffin three times at hourly intervals, before being imbedded. Usually two or three anthers from the same plant were imbedded in the same paraffin block and sectioned together.

The sections were cut at thicknesses of two or three microns. These thicknesses were chosen for two reasons: firstly, to keep the absorbancy down to a level between 0.5 and 1.0 since higher values would be less accurate; and secondly, to insure that the nucleoli were cut into slices whose upper and lower surfaces were of a diameter no smaller than 5.10 microns, which is the diameter of the area covered by the phototube aperture (see p. 351), so that the light that entered the phototube would not pass through any non-nucleolar part of the section. As can be shown, the diameter of the two surfaces of a 3-micron central slice of a nucleolus with a diameter of 6 microns (the majority of nucleoli measured had diameters larger than this) is 5.18 microns; that of a 2-micron central slice is 5.66 microns.

The sections were mounted on quartz slides with an adhesive made up of a 1% starch solution, which has no appreciable absorption in the ultraviolet. Sections whose ribonucleic acid contents were to be compared were always mounted on the same slide. The paraffin was removed from the sections by immersion in chloroform, followed by absolute ethanol and the slides were then transferred through 95%, 70% and 30% ethanols to water.

Blank slides.

Two types of agents are commonly used for extracting ribonucleic acids from tissue sections, namely, the enzyme ribonuclease and chemical agents such as hydrochloric acid, trichloracetic acid, perchloric acid, etc. A source of error in the use of ribonuclease is the frequent contamination with proteolytic enzymes. Crystalline ribonuclease, on the other hand, is expensive. Recently, perchloric

acid has been found to be very specific in the extraction of pentose nucleic acids at low temperatures, and of desoxypentose nucleic acids at elevated temperatures, and to have practically no proteolytic effects (ERICKSON, SAX and OGUR 1949, OGUR and ROSEN 1950, DI STEFANO 1952).

The blank slides, containing sections which were neighbors of those on the test slides, were treated with 10% perchloric acid at 4° C. for 18 hours. The test slides were left in distilled water for the same amount of time and at the same temperature. As a check on the completeness of the removal of ribonucleic acids, one set of slides was extracted for 30 hours, and the amount of reduction in the absorbance was found not to be significantly different from that obtained with an 18 hour treatment.

After the perchloric acid extraction, the sections were washed in distilled water several times and mounted in Farrant's medium, which is an aqueous solution of gum arabic and glycerine (GATENBY and BEAMS 1950) and consequently does not absorb to any appreciable extent in the ultraviolet.

IV. The microspectrophotometer.

The basic physical principles involved in spectrophotometry are simple. It has long been known that chemical structures absorb different amounts of electromagnetic radiations at different wavelengths. The characteristics of an absorption curve are thus often typical for a substance, so that a qualitative analysis is often possible. The height of an absorption curve, on the other hand, is related to the concentration of the chromophore (i.e., the structure responsible for the absorption) and the thickness of the absorbing layer (Beer-Lambert laws). The basic process in quantitative spectrophotometry, therefore, is the measurement of the amount of light transmitted by a substance at certain wavelengths. The union of the procedures of spectrophotometry and microscopy for the characterization and measurement of chemical components in cytological objects was due to CASPERSSON (CASPERSSON 1936, 1947, 1950; also see introduction).

It should be pointed out, however, that simple though the basic principles of microspectrophotometry are, the actual practice is fraught with a number of pitfalls. The most commonly mentioned source of error is the non-specific light loss due to scattering of light by large particles of different refractive indices in the specimen, and also to glare, i.e., light reflection and scattering by the optical components such as objective surfaces between the specimen and the detector. Such error can be reduced to a certain extent by the use of properly prepared blanks (POLLISTER and RIS 1947), which are used in the present investigation. An alternative way of correcting this error is to measure the loss of light in the specimen at a wavelength at which the specimen is believed to have practically no absorption, and then calculate the light loss by scattering at other wavelengths by assuming that the scattering varies inversely as the fourth power of the wavelength (CASPERSSON 1950).

Distributional error is another important source of error, since in most biological materials the absorbing substance is very rarely, if ever, evenly distributed throughout the structure as in a true solution. This error may be minimized by using fixing agents which cause the least amount of clumping or agglutination, and by studying the specimen at several different regions of the spectrum. The ultraviolet measurements were made with unstained material, so that the occasional presence of vacuoles, as seen in stained preparations, was not determined. The effect of such vacuoles on these measurements has not been evaluated.

Other sources of error include the effect of fixation, imbedding and sectioning, the effect of combination of the absorbing substance with other compounds, and the accuracy with which the thickness of the sections can be measured. Discussions of these problems are found in DANIELLI (1953), DAVIES and WALKER (1953), BLOUT (1953), and SWIFT (1953), and will not be attempted here. Suffice it to say that, despite the presence of a number of as yet not fully resolved problems in the theoretical and physical aspects of the instruments, microspectrophotometry has proven to be a research tool of great value to biologists. Together with a proper amount of caution in its application, a certain degree of optimism seems to be justified, especially if one is fully aware of the possible sources of error.

Description of the microspectrophotometer.

This instrument, which is diagrammed in Fig. 4, was assembled by Dr. CARL CLARK and is similar in conception to those described by POLLISTER and RIS (1947) and SWIFT (1950b and 1953). The xenon arc source (*A*) was cooled by an air blower. Occasional wandering of the arc about its electrodes impeded measurements. The source mirror (*B*) is an off-axis ellipsoid with a face diameter of 15 cm., f/4.5, especially made for this instrument by Mr. JEROME KAYE. Baffles were put in the monochromator (*D*) (Perkin-Elmer Corporation, Norwalk, Conn.,; Überlingen, Germany) to reduce stray radiation. The monochromator slits were set at 0.3 mm. The wavelength calibration was made using a mercury arc and anthracene (0.045 g./50 ml. cyclohexane) and benzene (0.046 ml./100 ml. cyclohexane) in 1 cm. silica cells. Although it would increase the available energy to match the monochromator aperture (f/4.5) to the quartz condenser input aperture (f/0.011) by means of a precondenser, this was not done. The reflecting objective (*J*) was screwed up in the microscope body tube to more closely approach the 160 mm. tube length for which the objective was designed. Instead of using an eye piece and projected image, the photomultiplier (*N*), in a Farrand tube housing, was mounted to replace the eye piece. The photomultiplier aperture (*M*), punched in metal foil, was set exactly in the objective image plane and made exactly conjugate in field of view to the side tube aperture (*L*), punched in transparent plastic. For measurements, nucleoli which were properly sectioned were centered by means of the mechanical stage in the side tube aperture (*L*), then the sliding prism (*K*) was removed to allow the radiation to fall onto the photomultiplier.

Section thickness was measured by focusing on the upper and lower surfaces of the section and reading differences on a fine adjustment screw scale designed by GLENN FRIEDMAN and Dr. CLARK. A plastic 360° protractor was glued onto the fine adjustment screw of the microscope, and the enlarged image of the protractor was projected onto a screen, marked with an index line, by means of a small lamp and convex lens. The device was calibrated in the working range of the fine adjustment screw. Its use reduced standard errors of the mean of a series of ten measurements on the same nucleolus from about 3.4% of the mean value when the fine

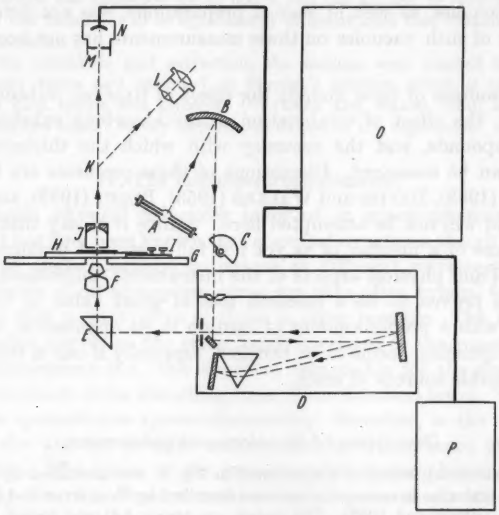

Fig. 4. Diagramshowing the light path and the main parts of the microspectrophotometer. A Xenon arc light source. B Mirror. C Light chopper. D Monochromator. E–N Microscope. E Substage prism of fused quartz. F Quartz condenser. G Stage. H Quartz slide and cover slip. I Mechanical stage. J Reflecting objective. K Sliding prism for directing light into L Lateral observation tube. M Photomultiplier tube diaphragm. N Photomultiplier tube. O Photomultiplier power supply. P Potentiometer and thermoocuple amplifier.

adjustment scale was read directly to about 1.5% when the optical fine adjustment scale was read. Measurements in the plane of the section were made with a Bausch and Lomb filar eye piece micrometer with a moving crosshair.

V. Experimental procedure.

From a number of absorption curves, the absorption peak was found to be in the neighborhood of 2637 Å. Unless otherwise specified, all measurements of the amount of ribonucleic acids were taken at this wavelength.

I. The following procedure was followed in each measurement on a single nucleolus.

1. A nucleolus whose upper and lower cut surfaces were larger than the aperture in the transparent diaphragm in the eyepiece of the side tube was selected and centered, with the monochromator set to the visible part of the spectrum.

2. The monochromator was set back into the ultraviolet, and the sliding prism in the microscope tube was moved to allow the light to pass into the phototube.

3. The condenser was focused until maximum deflection was obtained in the potentiometer. Since the condenser was made of fused quartz, it had to be refocused for each different wavelength and therefore when absorption curves were to be taken, they had to be done point by point despite the fact that a recording potentiometer was provided. However, when a large number of measurements were taken at the same wavelength, there was usually no need to refocus after each measurement.

4. By means of the fine adjustment screw, the objective was focused until minimum deflection was obtained in the potentiometer. As the objective went into focus on the nucleolus and out of it again, the deflection went from a peak position through a depression and into another peak position. The reading at the depression, or minimum deflection, was taken as I.

Usually, only minor adjustment in the focus of the condenser and objective was necessary in going from one nucleolus to another on the same slide.

5. By means of the mechanical stage, the nucleolus was moved away and a blank part of the slide measured without changing the position of either the condenser or the objective. This second reading was designated as I'.

6. The transmittance (T) and absorbance (A) of the nucleolus were calculated from the following relationships:

$$T = I/I'; \qquad A = \log_{10} 1/T.$$

7. The nucleolus was recentered, and steps 4 through 6 were repeated at least three times for each nucleolus measured, and the A values thus obtained were averaged. There was usually good agreement among the different readings, the deviation from the mean being generally less than 5%.

8. The monochromator was changed back to the visible, and by viewing through the side tube, the objective was focused ten times on the upper surface of the nucleolus and ten times on the lower surface. The thickness (b) of the nucleolar slice was obtained by subtracting the mean of the second group of readings from that of the first. It should be pointed out that the actual thickness of the section thus measured could sometimes be as much as 0.5 microns more than the nominal thickness given on the microtome.

9. With the objective focused at a level at which the nucleolus appeared largest, ten diameter readings each were taken along two mutually perpendicular directions. The radius (r) of the nucleolus was obtained by halving the mean of these readings.

10. The actual amount of ribonucleic acids (R_a) per nucleolus is given by the relationship

$$R_a = \tfrac{4}{3}\pi r^3 \cdot c, \tag{1}$$

where c is the concentration of RNA in the nucleolus.

According to the Beer-Lambert laws,

$$A = a\,b\,c \quad \text{or,} \quad c = A/a\,b, \tag{2}$$

where a is the absorptivity and b the thickness of the section.

Substituting (2) into (1), we obtain

$$R_a = \tfrac{4}{3}\pi r^3 \cdot A/a\,b. \tag{3}$$

Since the value $4\pi/3a$ is a constant, a relative measure of the total amount of RNA per nucleolus, E_r, in arbitrary units, may be obtained from the formula

$$E_r = A\,r^3/b. \tag{4}$$

II. From 10 to 15 nucleoli were measured for each of the strains under investigation, and their E_r values averaged. The same was done for the same strain

on the blank slide. The relative amount of RNA per nucleolus, Rr, in arbitrary units, is given by the formula

$$R = \overline{E}_r \text{ test} - \overline{E}_r \text{ blank}. \tag{5}$$

III. The value R_r can be readily converted into absolute units by being multiplied by the factor $4/\pi 3a$:

$$R_a = R_r \ (4\,\pi/3\,a), \tag{6}$$

where

$$a = 2.0 \times 10^{12}\,\mu^3/\text{gm.}\dagger.$$

However, since the a value was calculated on the basis of a statistical tetranucleotide base content and was at best only approximate, the results will be given mostly in relative units, according to formula (5). R_a will be used only where it is desirable to show the order of magnitude of the absolute amount of RNA. If all errors are of the multiplicative type, it may be that ratios of either R_r's or R_a's are more accurate than the values themselves. But it should not be assumed that the use of the term "relative" makes all considerations of errors unnecessary.

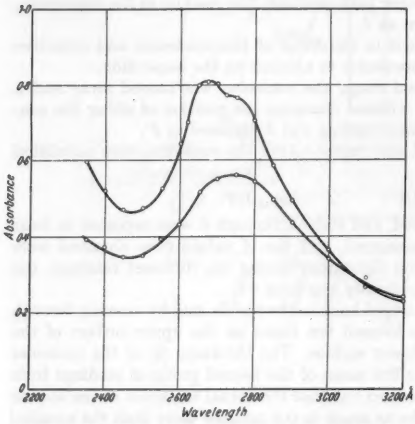

Fig. 5. Ultraviolet absorption spectra of early pachytene nucleoli showing the effect ofperchloric acid extraction. The lower curve is from a nucleolus on a slide treated with a 10 % perchloric acid at 4° C. for 18 hours; the upper curve from a neighboring section of another nucleolus from the same anther on a slide left in distilled water for the same period of time and at the same temperature.

VI. Results.

General composition.

The ultraviolet absorption curves of the nucleolus in the microsporocytes in maize (Figs. 5–11) are similar in general to those of the nucleoli in other organisms, such as *Drosophila*, the sea urchin, and spinach (CASPERSSON and SCHULTZ 1940). The spectra are dominated by a pronounced hump around 2800 Å, indicating the presence of a large amount of proteins besides the ribonucleic acids. There is, however, no appreciable displacement of the protein peak towards the longer wavelengths, as reported by the Caspersson school.

Absorption spectra of nucleoli from sections treated with perchloric acid at 4° C. for 18 hours and of nucleoli from control sections kept in distilled water at the same temperature and for the same length of time

† Calculated by Dr. CLARK on the basis of a statistical tetranucleotide base content, according to data given in CASPERSSON (1950), p. 65.

are shown in Fig. 5. It is evident that the nucleic acid peak is completely removed by the perchloric acid treatment, and a broad absorption band around 2750 Å due to the proteins remains. The absorbance at 2637 Å was reduced to approximately one half by the cold perchloric acid treatment (Tables 2–5), a result in close agreement with that of

Table 1. *Diameter in microns, and amount of RNA (R_a), in absolute units, in the nucleoli of different strains of maize at early pachynema.*

	Strain	No. measured	Diameter	R_a
1	(KYS)[1], plant 1	10	5.87 ± 0.03	14.22×10^{-12} gm.
	plant 2	10	5.89 ± 0.06	14.50×10^{-12} gm.
	plant 3	10	5.90 ± 0.06	14.55×10^{-12} gm.
2		14	6.48 ± 0.08	7.35×10^{-12} gm.
3		12	6.43 ± 0.05	8.50×10^{-12} gm.
4		15	6.40 ± 0.10	10.32×10^{-12} gm.
5[2]		12	6.41 ± 0.12	13.50×10^{-12} gm.
6	"	14	5.87 ± 0.03	12.07×10^{-12} gm.

[1] A strain widely used cytologically because of the distinct morphology of the chromosomes and the ease with which they can be spread at pachynema.

[2] Heterozygous for the gene elongate (el).

POLLISTER and LEUCHTENBERGER (1949), who used ribonuclease and hot and cold trichloracetic acid for the extraction of RNA. A consistent although very slight shrinkage of the nucleoli subjected to the cold perchloric acid treatment is evident in all the data collected.

The quantitative data show a remarkable degree of constancy in the amount of RNA present in each nucleolus of the same stage of division. This is true not only for cells of the same plant, but also for sister plants of the same strain. However, there seems to be considerable strain-specific difference among the six strains which were used (Table 1).

Development of the nucleolus in microsporogenesis.

The nucleolus found at the beginning of the first meiotic prophase is a relatively small spherical structure which undergoes a period of growth during the long leptotene stage, during which the chromosomes are in a slender and attenuated condition. There is as a rule only one nucleolus, with which both organizers of the diploid complement are associated, usually at different points. The maximum size of the nucleolus is attained in mid-pachynema (Table 2) and thereafter it begins to decrease in size. This shrinkage coincides with a rapid increase in the basophilia of the chromosomes. At late diakinesis, when the last remnant of the nucleolus disappears, the chromosomes are in the form of very short and thick, deep-staining bodies. The nucleolus reappears

transiently in telophase I and interphase, often giving the appearance of an irregular conglomeration of the matrix material which envelopes the chromosomes during these stages. Because of their small size and irregular outline, the nucleoli of these stages were not included in the measurements. The nucleolus disappears again during prophase II, and reappears in telophase II. Each number of the spore quartet has a single nucleolus, which could not be measured with the present instrument because its diameter is much smaller than that of the phototube diaphragm aperture.

The amounts of RNA at the different stages of the first prophase are given in Table 2, and the changes in the size and RNA content

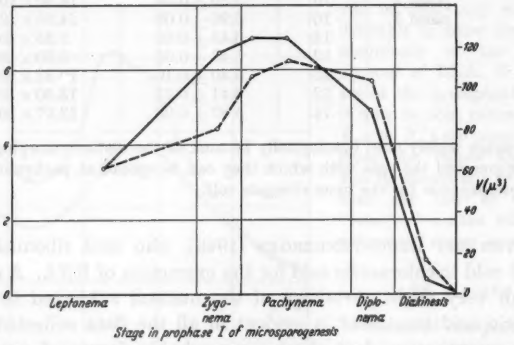

Fig. 6. Changes in the amount of RNA, R_r (solid line), in the nucleolus, and in the volume (broken line) of the nucleolus during prophase I of microsporogenesis. Data taken from Table 2.

undergone by the nucleolus represented graphically in Fig. 6. It is clear that the increase in the volume of the nucleolus lags behind that of the RNA content. This might be taken as an indication that the incorporation of proteins and other components into the nucleolus is a process which follows and is governed by the RNA. This interpretation finds confirmation in the finding that there is a slight but consistent increase in the RNA/protein ratio during prophase I until diplonema, as is evident from the absorption spectra of nucleoli of the different stages of prophase I shown in Fig. 7.

Another significant point that emerges from these data is that there is a doubling of the amount of RNA at some point between leptonema and zygonema. This doubling, on the one hand, coincides with a doubling of the volume of the nucleolus during the same period, and on the other hand, closely follows the reduplication of the chromonemata and the doubling of the amount of desoxyribonucleic acids (DNA) which occurs

during leptonema (SWIFT 1950a). This doubling of the amount of RNA in the nucleolus may be the result of the doubling of the amount of DNA in the nucleus, or it may be attributed to the reduplication of the nucleolar organizer. The second alternative seems to be the more likely one in the light of the other results obtained, which will be discussed in another section. The amount of RNA stays

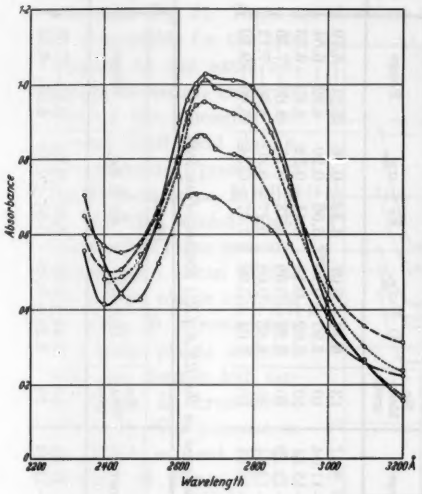

Fig. 7.

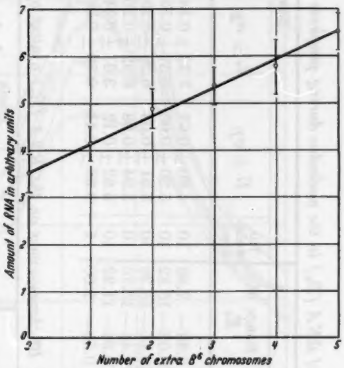

Fig. 8.

Fig. 7. Absorption spectra of nucleoli in different stages of prophase I of microsporogenesis: leptonema (————), early pachynema (--------), late pachynema (.....), diplonema (—·—·—), and diakinesis (—··—··—).

Fig. 8. Relationship between RNA content in the nucleolus and the number of extra nucleolar organizers. Vertical lines represent standard errors.

fairly constant through zygonema and pachynema; during diplonema and diakinesis there is a gradual drop concomitant with the shrinkage in the size of the nucleolus.

The effect of extra nucleolar organizers.

Comparisons were made of the amount of RNA in nucleoli of plants carrying from 0–5 B^6 chromosomes. The amounts of RNA (expressed in arbitrary units) per nucleolus in early pachytene microsporocytes with different numbers of B^6 chromosomes are summarized in Table 3. As was evident from MCCLINTOCK's work (1934), the functional activity of the nucleolar organizer does not depend upon its structural integrity. In the B^6 translocation used in the present studies, each one of the B^6 chromosomes, which carries a portion of the nucleolar organizer,

Table 2. *Changes in the diameter, volume, and amount of RNA (R_r) in the nucleolus during prophase I of microsporogenesis.*

Stage	Test					Blank				
	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	V μ^3	$\bar{E}_r \pm \sigma_{\bar{E}_r}$	Range of $\bar{E}_r$	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E}_r \pm \sigma_{\bar{E}_r}$	Range of $\bar{E}_r$	R_r
Leptonema	15	4.89 ± 0.05	61.2	6.70 ± 0.22	5.88— 7.88	10	4.80 ± 0.02	3.32 ± 0.14	2.87—3.86	3.38 ± 0.26
Zygonema	10	5.38 ± 0.07	81.6	12.98 ± 0.80	10.02—15.82	10	5.20 ± 0.06	6.57 ± 0.33	5.80—7.70	6.41 ± 0.86
Early Pachynema	12	5.89 ± 0.06	106.9	13.17 ± 0.54	10.14—15.36	10	5.51 ± 0.03	6.28 ± 0.28	5.02—7.23	6.89 ± 0.61
Mid Pachynema	15	6.02 ± 0.04	114.3	13.64 ± 0.38	12.44—15.76	10	5.94 ± 0.08	6.82 ± 0.20	6.01—7.97	6.82 ± 0.43
Diplonema	10	5.85 ± 0.32	104.8	8.64 ± 0.95	6.94—11.92	10	5.62 ± 0.12	3.81 ± 0.34	3.29—6.30	4.83 ± 0.76
Diakinesis	8	3.32 ± 0.35	19.2	1.10 ± 0.15	0.50— 2.00	5	3.35 ± 0.41	0.70 ± 0.06	0.48—0.82	0.40 ± 0.16

Table 3. *Effect of increasing number of B^6 chromosomes on the RNA (R_r) content of nucleoli.*

Chromosomal Constitution	Test				Blank				R_r: $\bar{E}_{r_{test}} - \bar{E}_{r_{blank}}$
	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E}_r \pm \sigma_{\bar{E}_r}$	Range	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E}_r \pm \sigma_{\bar{E}_r}$	Range	
2N	14	6.48 ± 0.08	7.90 ± 0.37	5.85— 9.67	13	6.14 ± 0.07	4.39 ± 0.16	3.21—5.79	3.51 ± 0.40
2N + B^6	13	6.51 ± 0.04	9.72 ± 0.31	8.49—11.04	10	5.97 ± 0.10	5.56 ± 0.20	4.82—6.54	4.16 ± 0.37
2N + 2B^6	13	6.39 ± 0.08	10.29 ± 0.34	8.60—13.61	10	6.02 ± 0.16	5.41 ± 0.22	4.40—6.33	4.88 ± 0.40
2N + 3B^6	12	6.64 ± 0.09	11.09 ± 0.36	9.25—12.53	10	6.29 ± 0.09	5.73 ± 0.27	4.86—7.55	5.36 ± 0.45
2N + 4B^6	10	6.85 ± 0.14	11.92 ± 0.51	9.60—13.44	10	6.53 ± 0.08	6.17 ± 0.30	4.90—7.12	5.75 ± 0.59
2N + 5B^6	15	7.40 ± 0.09	13.86 ± 0.20	12.55—15.61	10	7.04 ± 0.09	7.37 ± 0.34	5.90—9.49	6.49 ± 0.39

Table 4. *Effect of B chromosomes on the RNA (R_r) content of nucleoli*

Chromosomal Constitution	Test				Blank				R_r: $\bar{E}_{r_{test}} - \bar{E}_{r_{blank}}$
	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E}_r \pm \sigma_{\bar{E}_r}$	Range	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E}_r \pm \sigma_{\bar{E}_r}$	Range	
2N	12	6.43 ± 0.05	10.39 ± 0.31	8.72—12.55	10	6.44 ± 0.08	6.33 ± 0.21	5.77—7.66	4.06 ± 0.37
2N + 8B	15	6.49 ± 0.06	10.60 ± 0.27	9.01—12.54	10	6.28 ± 0.10	5.81 ± 0.20	4.55—6.36	4.79 ± 0.34

is capable of forming a part of the nucleolus. There is a practically linear relationship between the total amount of RNA in the nucleolus and the number of B^6 chromosomes present in the nucleus (Fig. 8). The B^6 chromosome carries, besides the fraction of the nucleolar organizer and the satellite of chromosome 6, the greater part of the B chromosome (see Fig. 2). There are therefore two possible sources which may be responsible for the production of the extra nucleolar material: the fraction of the nucleolar organizer itself, and the heterochromatin present in the B chromosome. In order to test the second possibility, absorption measurements were taken of nucleoli from plants carrying 8 extra B chromosomes and from plants isogenic with the former but carrying no B chromosome (Table 4). The increase in the RNA content in the nucleoli of plants of the former type was 4.79—4.06 = 0.73, or 18% of the amount present in the nucleoli of the latter type. The percentage increase in RNA content attributable to each B chromosome is therefore 18%/8, or only 2.3%. The average increase in RNA content due to the presence of each extra B^6 chromosome is, on the other hand, according to Table 3 (6.49–3.51)/5, or 0.6, an amount equivalent to 0.6/3.51, or 14% of the amount found in the diploid sibs. It is therefore evident that the nucleolar organizer of the B^6 chromosome by itself is capable of synthesizing extra nucleolar material, although the presence of extra heterochromatin in the form of B chromosomes is also capable of increasing the mass of the nucleolus but to a much smaller extent.

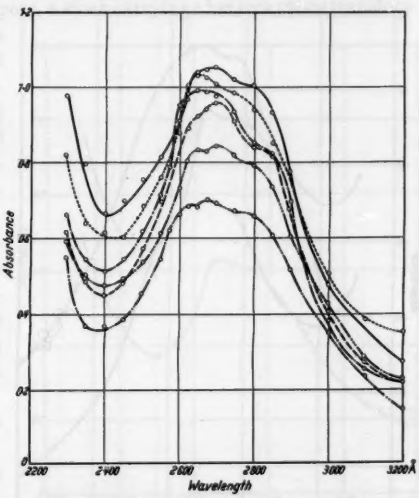

Fig. 9. Ultraviolet absorption spectra of nucleoli from plants carrying no B^6 chromosome (————), one B^6 chromosome (·····), two (.....), three (—·—·—), four (—··—··—), and five B^6 chromosomes (—···—···—). (Uncorrected for blank absorbance or thickness differences.)

The composition of the nucleolus does not seem to be affected by the presence of these extra B^6 chromosomes, as is evident from the shape of the absorption spectra of nucleoli of plants carrying different numbers of B^6 chromosomes (Fig. 9).

Nucleolar composition in plants trisomic for chromosome 6 and in triploids.

If the nucleolar organizer is indeed capable of synthesizing nucleolar material in the absence of, or independent of the presence of, extra euchromatin or heterochromatin, one would expect to find in nucleoli of plants trisomic for chromosome 6, since they have three nucleolar organizers instead of the two found in normal diploid plants, amounts of RNA and proteins which are three halves of those found in the normal diploid sibs. This was indeed found to be the case. Measurements of RNA in the nucleoli of a plant trisomic for chromosome 6 and in a normal diploid sibling are summarized in Table 5. The ratio of the amount of RNA per nucleolus in the trisomic plant to that in its diploid sib is 7.99/4.94, or just slightly over 3/2. It is clear that the presence of the extra chromosome 6, which amounts to less than one twentieth of the total amount of chromatin in a normal diploid nucleus, cannot be responsible for an increase of the amount of RNA of over one half.

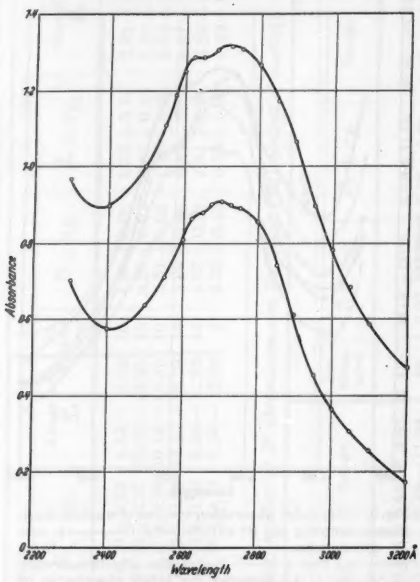

Fig. 10. Ultraviolet absorption spectra of nucleoli of a plant trisomic for chromosome 6 (upper curve) and its normal diploid sibling (lower curve).

That the synthesis of nucleolar material by the nucleolar organizer is not enhanced by the presence of extra euchromatin is further supported by the fact that in a triploid plant, the increase in the amount of RNA in the nucleolus is also only one half of the amount found in nucleoli of its diploid sib (Table 6). Compared to the trisomic plant, the triploid plant has nine more chromosomes, and yet the amount of RNA in the nucleolus is increased only to the same level as in the trisomic plant, and no higher.

The amount of proteins in the trisomic and the triploid plants is correspondingly increased, since the absorption spectra of the nucleoli from these plants maintain the same general shape as those from diploid siblings (Figs. 10 and 11).

The effect of chromosome rearrangement involving chromosome 6.

The data presented so far indicate that the composition of the nucleolus depends primarily on the nucleolar organizer and is relatively independent of extra chromatin. It was deemed of interest, however, to ascertain whether or not the function of the organizer is affected by the chromosome segment with which it is associated, namely, the rest of chromosome 6. For this purpose, a stock carrying a heterozygous translocation involving chromosomes 6 and 2 was procured and selfed. In the F_1 generation, sib plants carrying the normal chromosomes 6 and the translocated chromosomes 6^2 in heterozygous and homozygous conditions were obtained. Even though the total amount of chromatin and the number of nucleolar organizers remain the same in these plants the length of the chromosome associated with the organizer differs markedly (see Fig. 3). Table 7 shows that the amounts of RNA found in the nucleoli were quite different. Although the number of nucleoli measured is relatively small, there is a very strong indication of a discontinuous variation in the amount of RNA in all the three types of sib plants (Table 7, Fig. 12). These RNA values cluster around means which seem to fall into a simple multiple series of 2:3:4. In the plant carrying two chromosomes 6 the values fall mostly in the lowest class; in the plant carrying the translocation in the heterozygous condition, the values are divided about equally between the lowest and middle classes; while in the plant carrying the translocation in the homozygous condition, the values fall mostly into the middle and highest classes.

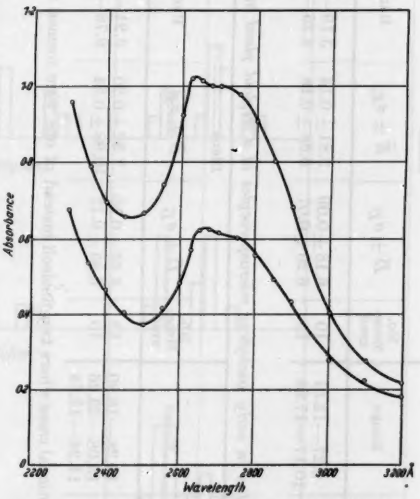

Fig. 11. Ultraviolet absorption spectra of nucleoli of a triploid plant (upper curve) and its diploid sibling.

There seems to be no question that the occurrence of nucleoli whose RNA content falls into the higher classes is associated with the length of the chromosome segment associated with the nucleolar organizer (Fig. 3). The length of the chromosome 6^2 at pachynema is approximately one half longer than that of the normal chromosome 6. The total length

Table 5. *Amount of RNA (R_r) per nucleolus in early pachytene microsporocytes of a plant trisomic for chromosome 6 and its normal sib*

Chromosomal Constitution	Test				Blank				R_r: $\bar{E}_{r_{test}} - \bar{E}_{r_{blank}}$
	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E} \pm \sigma_{\bar{E}_r}$	Range	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E} \pm \sigma_{\bar{E}_r}$	Range	
2N	15	6.40 ± 0.10	8.75 ± 0.43	6.27—12.51	10	6.18 ± 0.09	3.81 ± 0.14	3.19—4.32	4.94 ± 0.63
2N+6	15	6.34 ± 0.07	13.97 ± 0.73	10.17—17.98	10	6.20 ± 0.07	5.88 ± 0.18	4.25—6.24	7.99 ± 0.75

Table 6. *Amount of RNA (R_r) per nucleolus in early pachytene microsporocytes of a triploid plant and its diploid sib.*

Chromosomal Constitution	Test				Blank				R_r: $\bar{E}_{r_{test}} - \bar{E}_{r_{blank}}$
	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E} \pm \sigma_{\bar{E}_r}$	Range	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E} \pm \sigma_{\bar{E}_r}$	Range	
2N	12	6.41 ± 0.12	13.67 ± 0.74	10.28—16.90	12	6.25 ± 0.10	7.32 ± 0.20	6.21— 9.05	6.35 ± 0.75
3N	11	7.13 ± 0.05	20.38 ± 1.07	15.08—26.26	10	6.95 ± 0.17	10.98 ± 0.24	8.78—12.15	9.40 ± 1.11
3N [1]	3	6.47 ± 0.16	12.69 ± 0.61	11.24—13.74					

[1] These three nucleoli might represent the exceptional cases where two nucleoli instead of one were formed and the smaller one was not included in the section.

Table 7. *Amount of RNA (R_r) per nucleolus in early pachytene nucleoli of microsporocytes of plants carrying the normal chromosomes 6 and the chromosome 6^2 in heterozygous and homozygous conditions.*

Chromosomal Constitution	Test				Blank				R_r: $\bar{E}_{r_{test}} - \bar{E}_{r_{blank}}$
	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E}_r \pm \sigma_{\bar{E}_r}$	Range	No. Measured	$\bar{D} \pm \sigma_{\bar{D}}$	$\bar{E} \pm \sigma_{\bar{E}_r}$	Range	
2, 2, 6, 6	10	5.87 ± 0.03	11.86 ± 0.40	9.79—13.64	10	5.79 ± 0.05	6.10 ± 0.30	4.92— 7.04	5.76 ± 0.50
	4	6.10 ± 0.05	17.17 ± 0.39	16.20—17.71	5	5.95 ± 0.06	9.03 ± 0.40	8.23—10.41	8.14 ± 0.56
$2, 2^6, 6, 6^2$	7	6.48 ± 0.09	11.43 ± 0.65	9.60—13.45	8	6.24 ± 0.08	5.92 ± 0.28	4.41— 6.92	5.51 ± 0.70
	10	6.80 ± 0.07	17.66 ± 0.29	15.51—21.58	7	6.56 ± 0.07	9.12 ± 0.35	8.03—11.26	8.54 ± 0.45
$2^6, 2^6, 6^2, 6^2$	4	5.96 ± 0.05	11.39 ± 0.95	9.05—14.33	5	5.81 ± 0.05	5.71 ± 0.37	4.96— 6.90	5.68 ± 1.02
	9	6.48 ± 0.09	18.36 ± 0.31	17.00—19.44	8	6.12 ± 0.03	9.51 ± 0.25	8.33—10.92	8.85 ± 0.76
	4	7.14 ± 0.13	24.04 ± 0.41	23.20—25.24	2	6.80 ± 0.20	13.20 ± 0.16	12.98—13.42	10.84 ± 0.44

of chromosome segments associated with the nucleolar organizers in plants heterozygous for the translocation is therefore about one fourth longer than that in the normal sibs, while that in plants homozygous for the translocation is about one half longer. The total lengths of chromosome segments associated with the nucleolar organizer in the

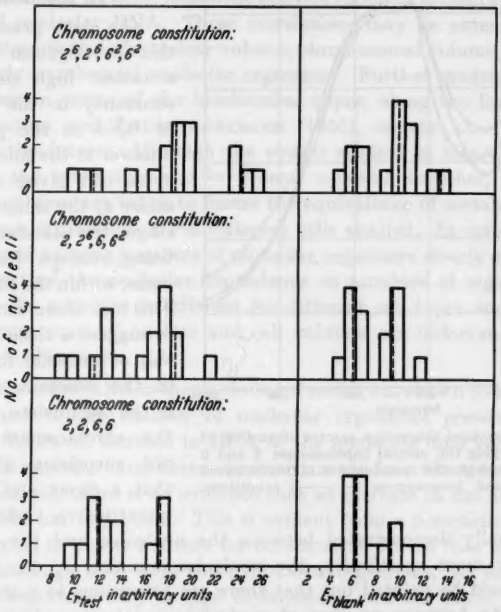

Fig. 12. Diagram showing discontinuous variation in the RNA content of nucleoli of sib plants obtained by selfing a plant heterozygous for the 2–6 translocation. Three classes of RNA values can be recognized on both the control (right column) and test (left column) slides. Mean values of each class are designated by broken lines.

three types of sib plants, i.e., the one with normal chromosomes 6, the one with the heterozygous translocation, and the one with the homozygous translocation, therefore fall into the ratio of $1:1\frac{1}{4}:1\frac{1}{2}$, or 4:5:6. Even though this ratio does not quite fit the ratio of 2:3:4 actually obtained, it is still highly suggestive that the length of the chromosome segment associated with the nucleolar organizer is at least partially responsible for the increase in the RNA content of the nucleolus. The reason why RNA values in these plants fall into distinct classes remains obscure.

Absorption spectra of early pachytene nucleoli of sib plants of the three types are shown in Fig. 13. The nucleic acid/protein ratio remains about the same in all three types.

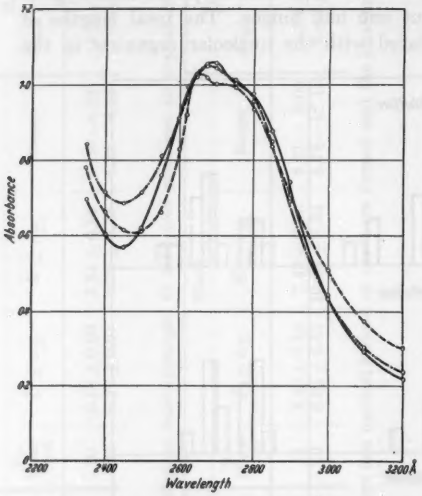

Fig. 13. Ultraviolet absorption spectra of nucleoli of siblings carrying the normal chromosomes 2 and 6 (————) and the 2–6 translocation in heterozygous (·····) and homozygous (— — —) conditions.

VII. Discussion.

The data presented in this investigation indicate a rather high degree of constancy in the amount of RNA in the prophase nucleoli of the microsporocytes of a given strain of maize at the same division stage, the standard errors being, except for a few cases, within the 10% level. This fact alone would seem to suggest a close relationship of nucleolar formation to the constant elements of the cell nucleus, namely, the chromosomes. It is not surprising, therefore, that a direct and clearcut quantitative relationship was actually demonstrated between the nucleolus and the chromosomes, or more specifically, the nucleolar organizer.

It should be pointed out that there are exceptions to a strict proportionality between number of nucleolar organizers and amount of RNA in the nucleolus. SCHRADER and LEUCHTENBERGER (1950) found that the lobes of the testis of *Arvelius albopunctatus* are characterized by different cell sizes with nuclear volumes of 200, 400 and 1600 cubic microns. Although the chromosomal constitutions of the large, normal-sized and small spermatocytes were identical and the amount of DNA approximately the same in all, both the total protein and RNA content of the nucleoli varied proportionately with the nuclear volume. Assuming a uniform biochemical turnover of these constituents, one may expect that these larger cells have higher metabolic activities than the smaller cells. One may note from this work by SCHRADER and LEUCHTENBERGER that the nuclear/nucleolar volume ratio is smaller for the large cells than for the small cells, supporting the concept that nucleolar volume varies more with metabolic activity than does nuclear volume.

CASPERSSON (1950) reviews other work showing increased nucleolar RNA with increased metabolic activity, and mentions the concomitant increase in nucleolar volume. The classical work relating nucleolar volume to metabolic activity does not include studies of RNA, although from CASPERSSON'S work a three-way correlation may be expected between nucleolar volume, metabolic activity (notably protein synthesis), and total nucleolar RNA. These correlations may be extended with modifications to include nuclear volume, chromosomal volume, and now particularly numbers of nucleolar organizers. Further studies of these correlations in terms of the biochemical types, along the lines begun by SCHRADER and LEUCHTENBERGER (1950), remain an important work for the future. Although the strains studied in this work were raised in the same field and in general collected together, excessive precautions were not taken to insure the equivalence of metabolic state at the time of fixation of the different cells studied. In spite of this, the effects of varying numbers of nucleolar organizers clearly stand out. It may be that the nucleolar dependence on numbers of organizers or on metabolic activities is different for different cell types and species. The measurements of nuclear and cell volumes are unfortunately not included in this work.

The amount of RNA in the nucleolus of maize was shown to be strictly proportional to the number of nucleolar organizers present in the nucleus. While an increase in RNA of the nucleolus does result when the nucleus contains extra heterochromatin in the form of supernumerary B chromosomes, there is no evidence that an increase in the amount of euchromatin has any effect. This is evident from a comparison of the RNA content in plants trisomic for chromosome 6 with that in triploid plants. Although both have three nucleolar organizers, the former has only one supernumerary A-type chromosome and the latter has ten, yet they did not have significantly different RNA contents. From this it would appear that it is the number of organizers which controls the composition of the nucleolus.

This does not mean, however, that euchromatin is not concerned in any way with the formation of the nucleolus. In the interchange involving chromosomes 6 and 2, whereby the length of the chromosome segment associated with the nucleolar organizer is lengthened, significant increases were found in the RNA content, although not the RNA/protein ratio, of the nucleolus.

The size of the nucleolus is in general roughly indicative of its RNA content. However, in some instances a large increase in the RNA content of the nucleolus is not reflected in a corresponding increase in its size (Tables 4 and 5). This may be the reason why certain workers,

e.g., DERMEN (1933) and RAMANUJAM (1937) failed to find any correlation between the degree of ploidy and nucleolar volume.

Further points of special interest that emerge from these studies are the following: (1) The function of the nucleolar organizer involves the actual synthesis of nucleolar material; the organizer is doing something more than merely serving as a pump or a reservoir for the collection and organization of the matrix material, or some other material produced by the chromosomes, into a single body. POLLISTER and LEUCHTENBERGER (1949), in their work on microsporocyte nucleoli of maize, found that cold trichloracetic acid extraction removed only 14% of the total absorbance at 2537 Å while hot trichloracetic acid extraction removed 52.8%. Since Carnoy fixation removed practically all the common acid-soluble mononucleotides, they believed that the 14% cold trichloracetic acid-soluble nucleotides which survived Carnoy fixation might be a polymer intermediate between a mononucleotide and a ribonucleic acid. The presence of this lower ribose nucleotide suggested to them the possibility that the nucleolus might be a site of synthesis of RNA. In the light of the results obtained in the present investigation, one would probably want to rephrase their statement by saying that the nucleolar organizer may be a site of synthesis of RNA. (2) The entire nucleolar chromosome is probably involved in some way in the synthesis of nucleolar material by the nucleolar organizer, so that its capacity for synthesis can be enhanced by the addition of an extra segment of chromatin to the nucleolar chromosome. (3) In the formation of the nucleolus, the RNA seems to be formed in advance of the proteins; in other words, the RNA could very well be the agent under whose influence the proteins are formed.

The last statement brings up the whole question of protein synthesis in the cell. Workers in this field are generally agreed that the sites of protein synthesis are particularly rich in RNA, although they differ as to the locality of these sites. CASPERSSON (1947, 1950) believed the center of protein synthesis to be inside the nucleus, in particular the nucleolar-associated chromatin and the nucleolus, while BRACHET (1950), 1952) emphasizes the importance of the cytoplasmic granules, especially the microsomes. ALLFREY, DALY and MIRSKY (1953) obtained direct evidence for the dependence of protein synthesis on the presence or intactness of RNA in the microsomes from the observation that the uptake of N^{15}-alanine by an *in vitro* system of microsomes and mitrochondria was impaired by preincubation of the microsomes with ribonuclease. On the other hand, there is good evidence that the nucleolus is also involved in protein synthesis. FICQ (1953), using an autoradiographic method based on the observation of individual particles, found that C^{14}-glycine was incorporated into the RNA and proteins of the

nucleolus of the oocyte of *Asterias* about 100 times more rapidly than into the cytoplasm. Further light was shed on the relationship between RNA and protein synthesis by PARDEE (1954) in his work on enzyme induction in strains of *Escherichia coli* which require purines, pyrimidines, or phosphate for growth. Within a few minutes of exhaustion of these compounds from the medium, induction of enzymes ceased. By inhibition of DNA synthesis with mustard gas, it was shown that DNA synthesis was not necessary for induction. It was therefore suggested that continuous formation of RNA was essential for protein formation. If the same hypothesis holds true in maize, it is not surprising to find that during the growth of the nucleolus in the early part of prophase I in the microsporocyte, the increase in the protein content trails that of the RNA content.

Summary.

1. The composition of microsporocyte nucleoli of maize with different chromosomal constitutions was studied by the analysis of absorption spectra obtained from an ultraviolet microspectrophotometer.

2. The presence of ribonucleic acids (RNA) and proteins in the nucleolus was confirmed by the presence of two broad and overlapping absorption peaks around 2637 Å and 2800 Å. The former peak was removable by treatment of the tissue sections with cold perchloric acid.

3. The amount of RNA per nucleolus was determined by measuring the absorbance and the diameter and thickness of nucleolus sections at 2637 Å. Non-specific light loss and absorption due to proteins were corrected for by the use of blank slides which were subjected to cold perchloric acid extraction.

4. By means of the above technique, the amount of RNA per nucleolus was found to be significantly different in the various strains of maize studied.

5. The size of and amount of RNA in the nucleolus were found to increase until mid-pachynema and then diminish and finally disappear at late diakinesis. The increase in volume was found to lag behind that in the RNA content. This observation, together with the observation that the RNA/protein ratio went down during the same period, was interpreted as indicating that during the growth of the nucleolus the RNA content increases faster than does the protein content, and that the synthesis or incorporation of proteins into the nucleolus is dependent upon RNA. The RNA content of the nucleolus was found to have doubled at some time between mid-leptonema and zygonema, an increase which was thought to be the result of the reduplication of the nucleolar organizer during leptonema.

6. A linear relation was established between the RNA content of the nucleolus and the number of extra nucleolar organizers present on supernumerary B^6 chromosomes. The extra organizers did not change the RNA/protein ratio of the nucleolus. Extra heterochromatin in the supernumerary B chromosomes was found to increase the RNA content of the nucleolus only very slightly.

7. Extra euchromatin was believed to have no appreciable effect on nucleolar composition since the RNA content of nucleoli from triploid plants, like that of nucleoli from plants trisomic for the nucleolar chromosome, was only three-halves as much as in their respective normal diploid siblings.

8. The entire nucleolar chromosome is probably involved in nucleolus formation since changes in the RNA content, though not in the RNA/protein ratio, were found in plants carrying a translocation involving the nucleolar chromosome.

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BEHAVIOR OF POLYTENE CHROMOSOMES OF RHYNCHOSCIARA ANGELAE AT DIFFERENT STAGES OF LARVAL DEVELOPMENT.

By

M. E. Breuer and C. Pavan.

With 20 figures in the text.

(Eingegangen am 23. Februar 1955.)

Introduction.

There is today a large amount of evidence showing that the chromosomes are genetically and morphologically differentiated along their length. Very little, however, is known about the behavior of these different parts of the chromosomes in different phases of the cell physiology. The principal changes which one can observe in the morphology of the chromosomes are those related to cell division. During the process of spiralisation (mitosis and meiosis) one can see visible differentiation of parts in a chromosome which expresses itself in formation of chromomeres, of constrictions and in heteropycnosis.

The polytene chromosomes, since their rediscovery by Heitz and Bauer (1933) and Painter (1933), proved to be favorable material for a study of the behavior of different parts of the chromosomes in different phases of cell physiology. The work of Bridges (1935, 1938), Painter (1941, Metz (1941), Poulson and Metz (1938), Dobzhansky (1944) and several others have shown that some sections of the polytene chromosomes appear sometimes as distinct discs, sometimes as capsules or heterochromatic discs and sometimes as "puffs". According to Painter (1941), "Salivary chromosomes are not static morphological structures, like metaphase chromosomes, but are constantly undergoing changes which involve differences in their physical and chemical make-up which, in turn, condition their responses to experimental treatments".

Kosswig and Şengün (1947), Kosswig 1948, Şengün and Kosswig (1948, Şengün (1949, 1951a, b) reached the conclusion that the disc patterns of the salivary chromosomes are variable, and a certain banding pattern is characteristic of only a special stage of the larval life. They concluded also that the disc patterns in chromosomes of *Chironomus* are not constant in different tissues. The conclusions of Kosswig and

Şengün were not confirmed by several cytologists including the authors of the present article [see Slizynsky (1950), Beermann (1952), Pavan and Breuer (1952), Bauer (1952a, b), Bauer and Beermann (1952), and Mechelke (1953)]. Beermann (1952), working with *Trichocladius vitripennis*, found that in this species some sections of the salivary gland chromosomes have different appearances in cells of different parts of the salivary gland. In one cell a section of the chromosome is represented by few bands while the same section of the chromosome in another cell of the same or different tissues could show an enormous "Balbiani ring" structure.

Breuer and Pavan (1952, 1953), working with *Rhynchosciara angelae*, have shown that enormous bulbs (structures similar to the "Balbiani Ring" found in *Chironomidae*) are formed at specific stages of the larval development. The development of some of these bulbs which start from clear euchromatic bands, pass through a stage of typical bulbous structure and return to the banded stage again can be clearly followed. These regions of the chromosomes behave differently in other tissues, even though the same bands can be seen in all. Mechelke (1953) working on *Acricotopus lucidus* has shown that sections of salivary gland chromosomes may appear as bulbs (Balbiani rings) which are in some tissues maintained from larvae to old pupae. In other parts of the salivary gland, however, the same section of the chromosome which shows bulbs in larvae, shows typical euchromatic bands in pupal stage.

Material and Method.

We used in this work larvae of *Rhynchosciara angelae*, collected in nature and brought to the laboratory to develop until the adult stage under more or less natural conditions. We collected larvae of different ages, some of which lived in the laboratory at room temperature (22° to 26° C) for a period longer than 30 days before pupation. These larvae were collected in "Fazenda Santa Cruz" at the station of Vila Atlântica (Railway Santos-Juquiá) in the State of São Paulo, Brazil.

Smears of salivary glands and of Malpighian tubules were prepared with aceto-orcein and examined in temporary slides or slides made permanent in diaphanol. For control, other fixatives such as formalin, were used. The material was also observed in physiological salt solution. The results were the same as with aceto-orcein although the preparations were less favorable for study.

Some Peculiarities of the Biology of *Rhynchosciara angelea*.

As was shown by Dreyfus et al (1951) *R. angelae* is a Sciarid whose larvae attain a length of 12—18 mm, and presents easily studiable polytene chromosomes in large salivary glands which are 12 to 18 mm

long (PAVAN and BREUER 1952 gave 10—15 mm as the larval length and 10—15 mm as the salivary gland length, but later measurements require the correction made above).

Polytene chromosomes present in the Malpighian tubules, as well as those present in some parts of the intestinal track, are as good as salivary gland chromosomes of many species of Drosophila. Besides the size of the larvae of the salivary gland and of the polytene chromosomes, two additional characteristics of these flies make them excellent material for study of the polytene chromosomes in different stages of larval life. These are: 1. the possibility of observing the band patterns of the polytene chromosomes in many stages of larval life, for at least 30 days before pupation; 2. as the gregarious larvae develop synchronously, one can sample the group at any one stage. Sampling the group every day, it is possible to follow the development of the group, which would correspond to following the development of a single individual by observing it every day.

Chromosomes of *R. angelae*.

As shown by PAVAN and BREUER (1952) the polytene chromosomes of the salivary gland of *R. angelae* are very convenient for cytological studies. They are very large, and good preparations in aceto-orcein can easily be obtained. This species has in its salivary glands four polytene chromosomes, of which three are single armed elements and one a large chromosome with two unequal arms. The two-armed chromosome was called A and the single armed ones B, C, and X respectively. PAVAN and BREUER (1952) made a comparative study of the banding pattern of chromosome A in salivary gland, in Malpighian tubules and in vesiculae seminales, and published a detailed map of this chromosome.

In the present work we will study in detail some parts of the chromosomes B and C. As shown in fig. 1, chromosomes B and X are about equally long, while chromosome C is the shortest in the group. A good recognition mark in the chromosome B is the block of heterochromatic material in the basal fifth of the chromosome at a point at which the chromosome is easily fractured in preparations. Close to the basal heterochromatic block there is a heavy disc which also looks like heterochromatin. The X chromosome can be easily distinguished from B by the absence of the above mentioned fracture point, by two instead of one heavy bands close to the basal heterochromatin and by the presence of a nucleolus at the base of the chromosome. This nucleolus, which is spherical in young larvae, looks like a dovetail figure made by hetero-

chromatic branches in the old larvae (see fig. 1). The chromosome C is the shortest of the group and is easily recognized by its base and tip which are shown in fig. 1.

Development of bulbs in Chromosome *B* and *C* of *R. angelae*.

The polytene chromosomes of *R. angelae* grow in diameter while the larva increases in size. When the larvae reach their full growth the

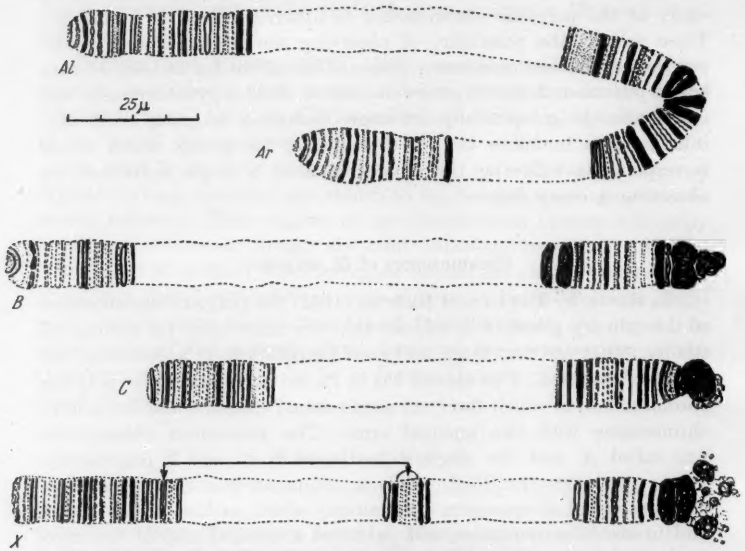

Fig. 1. Camera lucida drawings of the bases and distal end of the polytene chromosomes of the salivary gland of *R. angelae*. The chromosome A has two arms AL (left arm) and AR (right arm); the centromere region being the one which is folded. The arrows above chromosome X represent two points where there are repeated bands in this chromosome. The nucleolus regions is at the base of this chromosome. The scale represents 25 μ (see also fig. 5).

polytene chromosomes also reach their maximum width, and no longer increase in diameter. Observing the behavior of the tip of chromosome B, one can, however, see that some bands of this region grow in diameter and volume as represented in fig. 2. There, a section of this chromosome (fig. 2, a, b and c, section 2) which is represented by a typical euchromatic structure in young and full grown larvae, develops in a large bulb (puff), while the larva is preparing for pupation. This large bulb reaches its maximum size and later returns to the banded stage again. It hap-

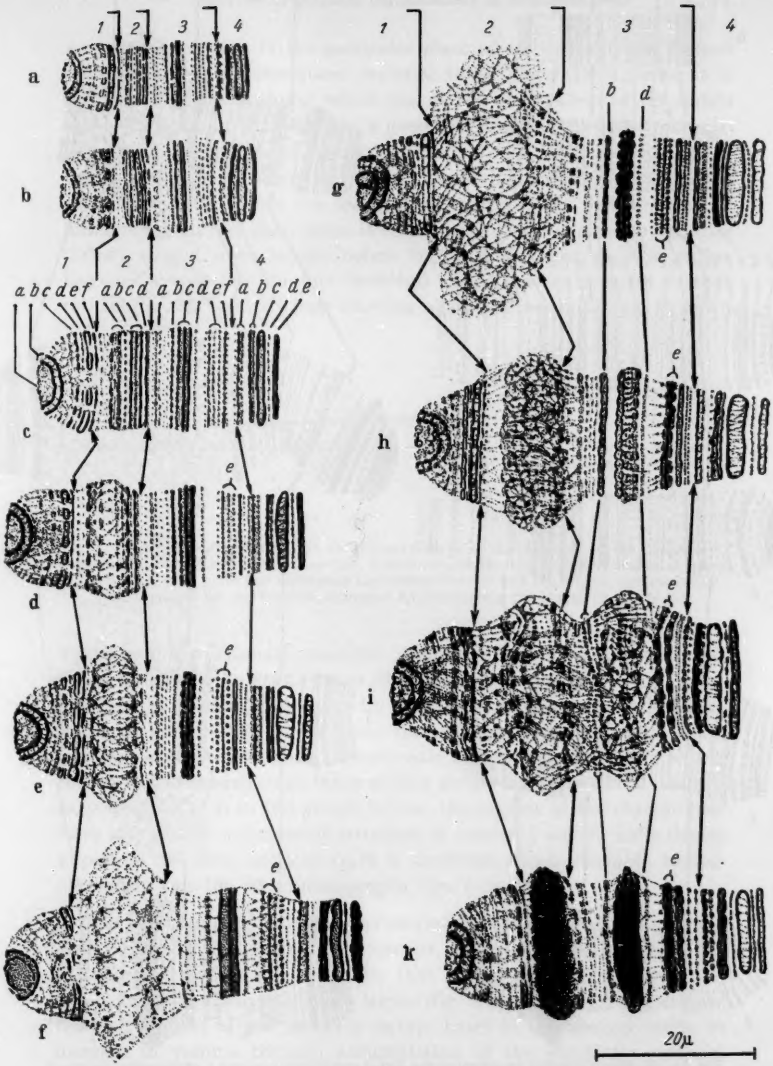

Fig. 2 a—k. Camera lucida drawings of the distal end of chromosome B in different stages of larval development. Phase c is a chromosome from a full grown larva; b is three days, and a eight days before c. Phases c to k represent about 8 days of larval growth (see fig. 4). The arrows and small letters indicate corresponding bands. The arabic numbers indicate sections into which the distal part of chromosome B was arbitrarily divided.

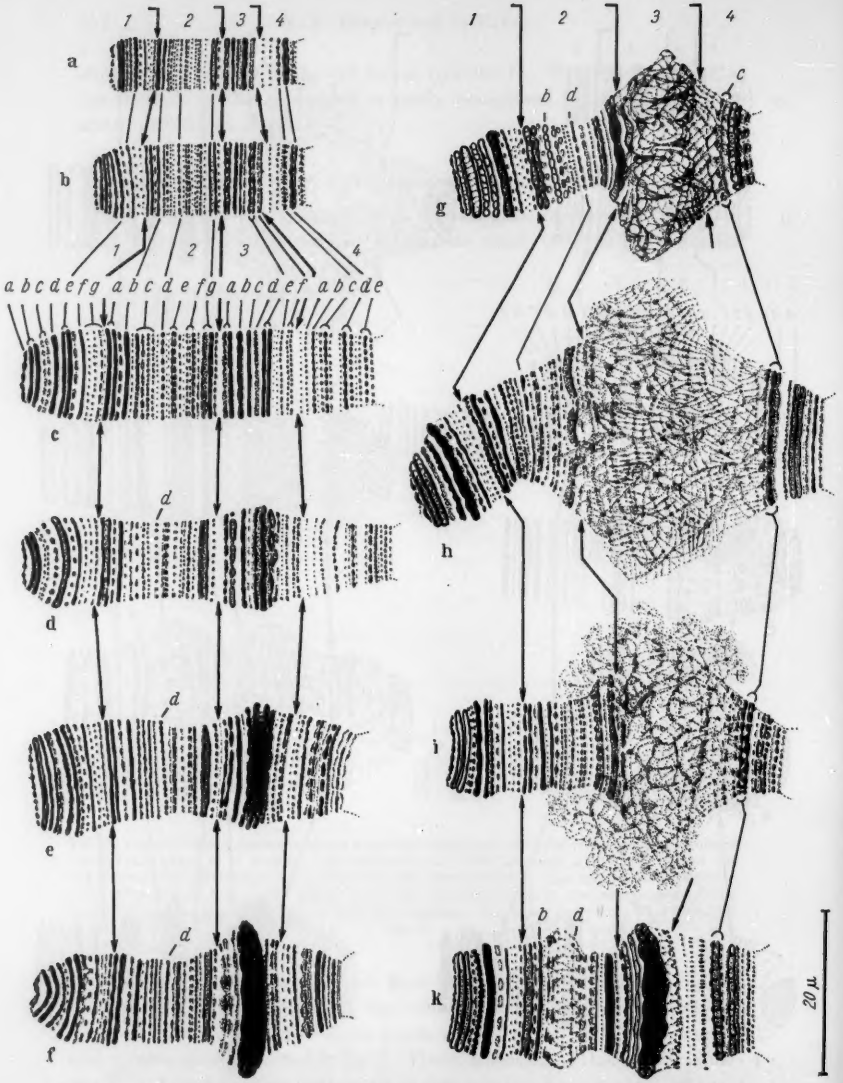

Fig. 3 a—k. Camera lucida drawings of the distal end of chromosome c of the salivary gland of *R. angelae*, in different stages of larval development. Phase c is from a chromosome of a full grown larva. Phase a is from a chromosome 16 days younger than the one in phases h and i. Phase b is from a chromosome eight days older than the one in phase a. Phases c to k represent eight days of larval development. The arrows and small letters indicate corresponding bands. The arabic numbers indicate sections into which this part of the chromosome was arbitrarily divided.

pens, however, that in the particular place where the bulb was formed an increase of the chromatic material takes place (DNA, since it is strongly Feulgen positive) which makes the observation of the bands rather difficult in that stage. In good preparations, however, one can see, beside the excess of the chromatic material the same number of bands as before the bulb formation. Observing section 3 of the same chromosome (fig. 2) one can see a similar process, but with some small differences. In this case, there is an increase of the chromatic material (DNA) around some bands, before the puffing; later on, this region becomes a bulb like the one described above, and subsequent to that returns to the banded stage showing again the accumulation of extra

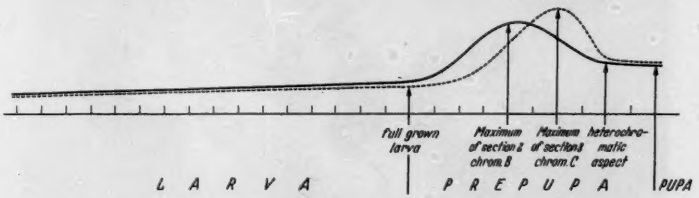

Fig. 4. A graphical representation in arbitrary units of the increase of the diameter of section 2 of chromosome B and section 3 of chromosome C in different stages of larval development. The plain line represents the chromosome B and the dotted, chromosome C. The divisions on the baseline represent approximately the length of the days.

amounts of the chromatic material. While the bulb in section 2 at room temperature (22° to 26° C) takes about five days to reach its full grow, the bulb in section 3 goes rapidly through the puff stage and returns to the banded stage. In this case the puffing is rapid, but the whole process, i. e., the formation of chromatic material, the puffing and the return to the banded stage takes as long as the one in section 2. As it is easy to see (fig. 2) in full grown larvae, the regions of the chromosome have a typically euchromatic structure in section 2 and 3, while during pupation the same sections have a structure which resemble heterochromatin (see the microphotographs figs. 6 to 19).

The same thing happens, as represented in fig. 3, to the tip of chromosome C during the larval development. Taking the section 3 in the chromosome of fig. 3 one will see that it has a typical euchromatic aspect from young to full grown larvae (fig. 3, A, B and C). Then, close to the pupation of the larvae a certain band in this section starts to increase in volume through accumulation of the chromatic material (DNA). This accumulation of chromatic material in section 3 normally reaches its maximum when the bulb of section 2 of chromosome B is fully grown. After this preliminary accumulation of the chromatic

material there occurs a kind of vacuolization and an enormous bulb develops. After the bulb formation this section returns again to the banded stage, but the band which accumulated chromatic material previously maintains its large volume.

The bulbs do not start to develop and do not reach their full growth synchronously in both chromosomes. The bulb in section 2 of chromosome B starts to develop a little earlier than the one in section 3 of

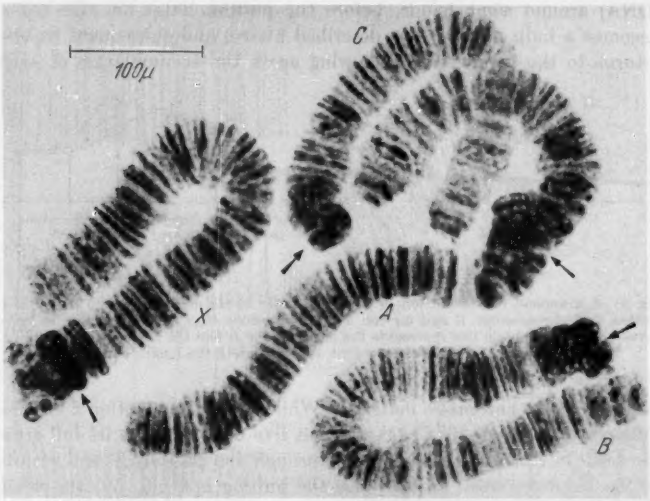

Fig. 5. Photomicrograph of the full set of polytene chromosomes of a full grown larva of *R. angelae*. The arrows indicate the centromere regions.

chromosome C. When the tip of chromosome B is in the phase J of figure 2, the section 3 of chromosome C will be in the phase H or I of figure 3. (For microphotography see fig. 6 to 20.) Figure 4 shows diagramatically the relation between the bulbs (section 2 of chromosome B

Fig. 6 to 12. Photomicrographs of the distal end of chromosome B of the salivary gland of *R. angelae* at different stages of larval development. Fig. 6 is from a young larva; Fig. 7 is from a chromosome of a full grown larva and fig. 12 from a chromosome of a larva in the stage between prepupa and pupa (see fig. 4). Fig. 11 is section 2 of this chromosome in its maximum bulb formation.

Fig. 13 to 19. Photomicrographs of the distal end of chromosome C of the salivary gland of *R. angelae* at different stages of larval development. Fig. 13 is from a chromosome of a full grown larva and fig. 12 is from a chromosome of a larva in the transitional stage to pupa (see fig. 4). Fig. 17 represents section 3 of this chromosome in its maximum bulb formation.

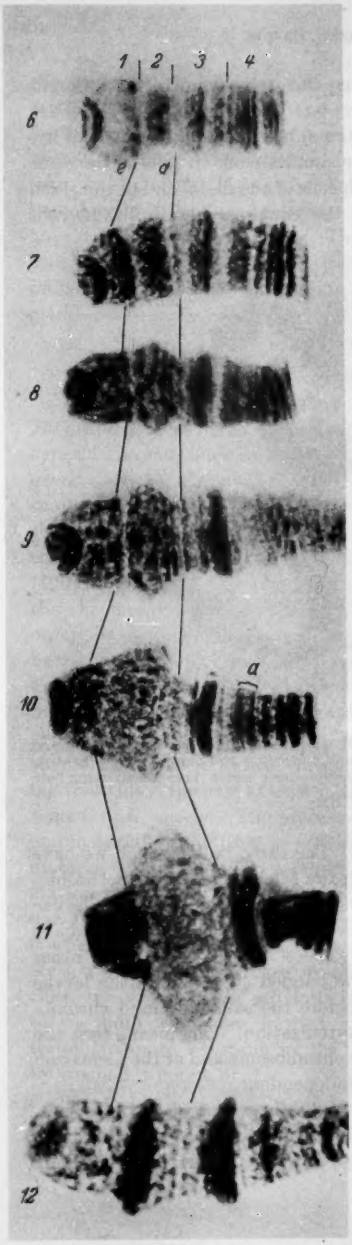

Fig. 6 to 12.

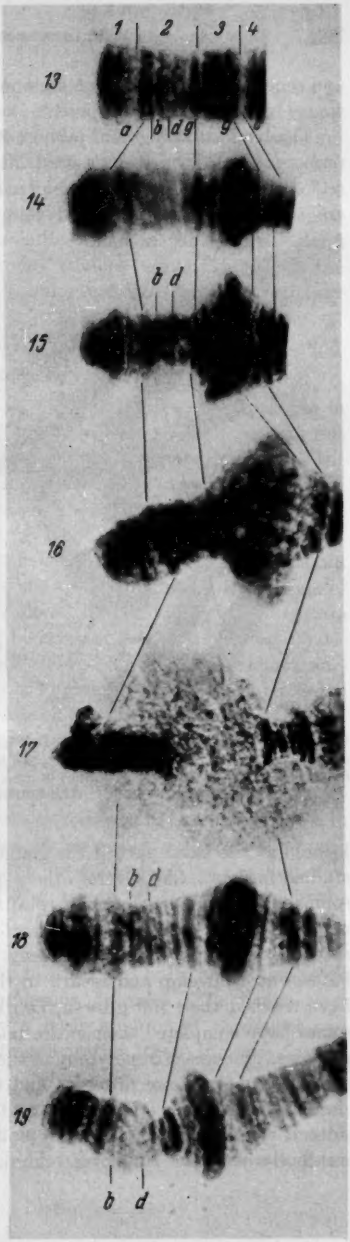

Fig. 13 to 19.

and section 3 of chromosome C) among themselves and to the different stages of the larval development.

The long salivary gland (almost 2 cms) has hundred of cells and the process of bulb formation is pratically simultaneous in cells of the same region of the two glands. The bulbs in cells of the distal end of the gland are somewhat more advanced than the correspondent bulbs in cells

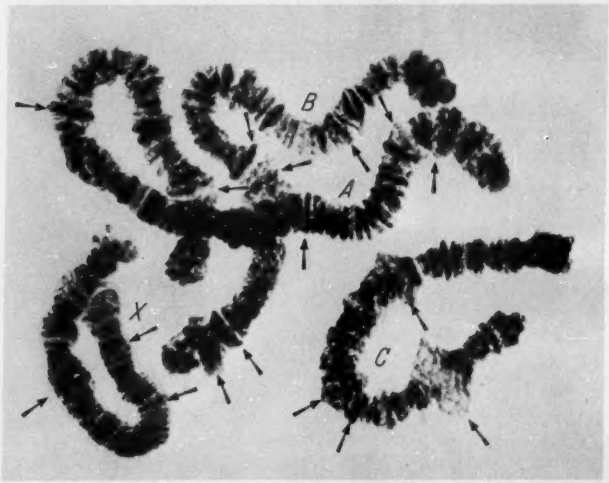

Fig. 20. Photomicrograph of the full set of polytene chromosomes of the salivary gland of a larva of *R. angelae* at the time when the bulb on section 3 of chromosome C is at its maximum. The arrows indicate regions of the chromosomes where there are stricking bulb formations.

located at the basal part of the gland. The three bulbs, which we have studied in detail, like several others in the same or in different chromosomes, have their development (puffing) after the time when the larvae reach their full growth and therefore after the chromosomes have reached their maximum of polyteny. There are, however, many other bulbs which develop and return to the banded stage before the larvae have reached their full growth, i.e., before the salivary gland chromosomes have completed their entire polytenization. This means that the processes of increase in polyteny of the chromosome and of the formation of bulbs (puffing) are different and independent.

Bulb formation is not peculiar to the salivary gland chromosomes, since it is observed in other polytenic chromosomes as well. We have seen bulbs in cells of Malpighian tubules, as well as in intestinal polytenic

chromosomes. It happens, however, that during the bulb formation in section 2 of chromosome B and section 3 of chromosome C, the homologous sections in the chromosomes of the Malpighian tubule cells are normal showing no visible transformations. On the other hand, when some bands of the Malpighian tubule cells are forming the bulbs, the homologous bands in salivary gland chromosomes do not show any striking transformations. The bulbs found in the salivary gland cells are not observed in Malpighian tubules cells. Conversely the bulbs formed in the Malpighian tubules are not found in salivary gland cells.

Discussion.

Since 1933, after the rediscovery of salivary gland chromosomes in *Bibio* and in *Drosophila*, a large amount of work has been done with this type of chromosomes to study the relation between the bands and the genes. There is a great deal of evidence showing that specific areas in the salivary gland chromosomes of *Drosophila* are, indeed, related to specific genes. Until now, however, there has been no evidence showing that these bands are functional units, as genes undoubtedly are. BEERMANN (1952) and MECHELKE (1953) have shown that some of the regions of the chromosome which appear as bulbs ("Balbiani rings") in a certain organ or sections of an organ may be represented by some euchromatic bands in another organ. MECHELKE showed also that the same transformation happens if one examines the chromosomes in the same organ in earlier and later stages of larval development. Although they do not claim that the bulb formation corresponds to the development of a single band of the polytene chromosomes, we have good evidence showing that this is the case at least in the bulb formed in section 3 of chromosome C of *R. angelae*. The process can be followed in this case because in our material the puffing is preceded by an accumulation of chromatic material in the band. In section 2 of chromosome B, as the bulb formation starts with a swelling of the region, the process might be due to the development of one or of several bands. In chromosome C when, after the accumulation of chromatic material, the band of section 3 starts to increase in size to form the bulb, the other bands close to it are break up into numerous granules. The bands close to the bulb which are formed by a large amount of chromatic material are more resistant to the fragmentation than the ones formed by a small amount of it.

The formation and the retention for a time of a new substance (which is Feulgen negative) inside and around the band is responsible for the bulb formation, and is also responsible for the increase in diameter of the

region of the chromosome. The compact chromatic material which accumulates in the band before the bulb formation is Feulgen positive; it breaks up gradually from large pieces to very small granules and thin threads within the bulb when the bulb reaches its full growth. These granules and threads continue to be Feulgen positive even when the bulb is at its maximum size. After a certain stage of development the Feulgen negative substance formed inside the bulb is eliminated from the chromosome and the correspondent regions of the chromosome return to the banded stage. The reverse process from the bulb to the band is very much like, if not identical, to the process of bulb formation. Studies are under way now to examine the substances which are produced during the bulb formation. Our preliminary experiments show that the Feulgen negative substance formed during the bulb development gives the same reaction with Methyl Green-Pyronin method as the nucleolus (it stains red). On the other hand, the same puffing region of the chromosome after the regression of the bulb does not stain red. Using the Methyl Green-Pyronin method one can see that several other regions of the polytene chromosome stain like the nucleolus, and in many cases we have the same reaction in regions of the chromosome which do not show any bulbs.

In the three bulbs we described above there occurs an accumulation of chromatic material after the puffing; as a consequence, exact identification of all bands of this stage of larval development is not easy. However, in good preparations one can count, beside the extra chromatic material accumulated, the same number of bands as before the bulb formations.

The bulb formation and return to the banded stage is a property of many bands. Some are as striking as the ones we described and many others are very minute. Surely this type of behavior of many bands in polytene chromosomes is related to the nucleolus formation. However, we think that this is the behavior of almost all (if not all) bands of the polytene chromosomes, and the behavior of the nucleolus organizer region is only a special case of a general process. The nucleolus of *R. angelae*, if formed at the basal end of the X chromosome and in salivary gland cell, is very diffuse and fragmented in old larvae and spherical and very evident in young ones.

As stated above, the relations between salivary gland chromosome bands and genes were studied by several authors (Painter 1934, Bridges 1935, Demerec 1941, Lewis 1945 and others). The experimental evidence suggests that a gene is located within a band. The behavior of the bands in polytene chromosomes described above appears to be morphological evidence of gene activities in these chromosomes. The bulb formation in a region of the chromosome at a certain time of larval

development is a manifestation of an activity of the respective genes at that time. Formation of a bulb at a certain locus of the polytene chromosome of the salivary gland cells, and absence of bulb in the corresponding locus of polytene chromosomes of the Malpighian tubules cells, is evidence of different gene activities in different tissues, as pointed out by BEERMANN (1952) and MECHELKE (1953) in midge flies. Such differences in the behavior of the same gene in different tissues should be kept in mind when discussing the problems of organic differentiation (STERN 1954).

Another point suggested by our data in *Rhynchosciara* is related to the theory of the constancy of the amount of DNA in all cells of a species [BOIVIN, VENDRELY and VENDRELY (1948), RIS and MIRSKY (1949), SWIFT (1950a, b), ALFERT and SWIFT (1953) and others]. The observations on bulb formation suggest to us that this theory may not hold in all cases. We observed an increase of the DNA content in certain bands (Feulgen reaction), which reaches an amount several times greater than that which the band had before going through the process. This increase of DNA is however not uniform along the length of the chromosome. There are some bands where the increase of DNA is very great while in some other bands there is no evidence of increase or decrease at all. The loci at which increases of DNA are observed are not the same in polytene chromosomes of different tissues. The increases of DNA in the regions which we have studied in detail (section 2 and 3 of chromosomes B and section 3 of chromosome C) are independent of the process of polytenization. The chromosome increases in diameter from young larvae until the time when they reach their full growth, and the bulb which we study in detail arises after this stage.

Bulb formation is not peculiar to *R. angelae*. *Chironomidae* have the so-called "Balbiani ring", which correspond exactly to the bulb in *R. angelae*. Several similar cases are known in *Drosophila* (BRIDGES 1935, DOBZHANSKY 1944, FREIRE MAIA 1953) in *Sciara* (POULSON and METZ 1938) and other Diptera. We are now studying another species of *Rhynchosciara*, *R. milleri*, which shows cycles of bulbs very similar to the ones found in *R. angelae*.

Summary.

Polytene chromosomes in cells of salivary gland, Malpighian tubules and intestine of *Rhynchosciara angelae* are very favorable for study. The polytene chromosomes of the salivary gland are among the largest available for cytogenetics work. The ones in Malpighian tubules and in some parts of the intestine are as large and as favorable for cytological studies as the salivary chromosomes of many species of Drosophila.

Two additional characteristics of *Rhynchosciara* make these flies excellent material for studies on the development of polytene chromosomes. 1. It is possible to observe the banding pattern of the polytene chromosomes at many stages of the larval life for at least 30 days before pupation, and 2. since the gregarious larvae develop simultaneously, one can sample the group at any stage desired. Sampling the group every day, it is possible to follow the development of the chromosomes as though one studied a single individual by observing it every day.

We have followed in detail the behavior of the bands in two sections of chromosome B and in one section of chromosome C, at different stages of larval development. Some regions of the chromosomes which are represented by typical euchromatic bands at one stage of the larval development may develop in enormous bulbs, and later on may return to the banded stage again.

The formation of the bulbs is not uniform in different sections of the same or of different chromosomes. In section 2 of chromosome B a certain locus swells enormously and then develops an enormous bulb, and later returns to the banded stage. At the point where the bulb was formed there is an accumulation of DNA, in amounts probably several times greater than before the bulb formation. In section 3 of chromosome B and section 3 of chromosome C the extra accumulation of DNA preceeds the formation of the bulb and is maintained during and after it. In the bulb formed in section 3 of chromosome C a single band seems to be responsible for the process.

As shown by several authors, experimental evidence suggests that a gene is located within a band. The bulb formation in polytene chromosomes may then be morphological evidence of gene activities. This type of bulb formations and of return to the banded stage is a property of many chromosomes bands, during larval development. This type of behavior of many bands in polytene chromosomes is related to the process of nucleolus formation. However, this behavior may be found in almost all (if not in all) bands of the polytene chromosomes. If so, the behavior of the nucleolus organizer region is only a special case of this general process.

The accumulation of DNA in different parts of the chromosome in cells of the same or of different tissues may be an argument against the theory of the constancy of the amount of DNA in all cells of a species. The bulb formations is not peculiar to *R. angelae* but occurs in several other Diptera.

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